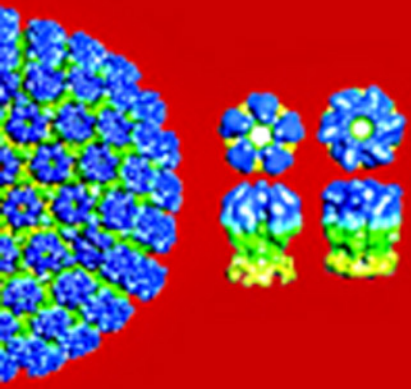
Human Herpesviruses

Biology, Therapy, and Immunoprophylaxis



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Human Herpesviruses

This comprehensive account of the human herpesviruses provides an encyclopedic overview of their basic virology and clinical manifestations. This group of viruses includes human simplex type 1 and 2, Epstein–Barr virus, Kaposi's Sarcoma-associated herpesvirus, cytomegalovirus, HHV6A, 6B, and 7, and varicella-zoster virus. The viral diseases and cancers they cause are significant and often recurrent. Their prevalence in the developed world accounts for a major burden of disease, and as a result there is a great deal of research into the pathophysiology of infection and immunobiology. Another important area covered within this volume concerns antiviral therapy and the development of vaccines. All these aspects are covered in depth, both scientifically and in terms of clinical guidelines for patient care. The text is illustrated generously throughout and is fully referenced to the latest research and developments.

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Preface

Diseases caused by the human herpesviruses were recognized by the earliest practitioners of medicine. Hippocrates, Celsus, Herodotus, Galen, Avicenna and others described cutaneous lesions typical of infections caused by herpes simplex viruses (HSV) 1 and 2, and varicella-zoster virus (VZV). 'Herpes,' the family name of these viruses, is traced to the Greek term for lesions that appeared to creep or crawl over the skin. Among the duties of John Astruc, physician to King Louis XIV, was to understand the diseases of French prostitutes, in Latin, the '*Puellae publicae*', which led to his description of *herpes genitalis*. Distinguishing between genital herpes and syphilis was an obvious concern in this social context as it is now. The modern scientific investigation of HSV can be dated to the work of Gruter, who first isolated the virus and demonstrated its serial transmission in rabbits. During the 19th century, experiments in human subjects showed that HSV and VZV could be transmitted from fluid recovered from HSV and VZV lesions. Demonstrating that Koch's posulates were fulfilled was important but arguably the truly revolutionary discovery about the herpesviruses was made by Andrews and Carmichael in the 1930s who showed that recurrent *herpes labialis* occurred only in adults who already had neutralizing antibodies against HSV. Since our modern understanding of all of the human herpesviruses revolves around latency and reactivation as established facts of their biology, it is important to remember that these concepts are far from obvious and to appreciate the creative insights of Doerr who proposed that recurrent HSV was not an exogenous infection but resulted from stimuli to the cell that caused the endogenous production of a virus-like agent and of Burnet and Williams who perfected the notion that HSV persists for life and "*remains for the most part latent; but under the stimulus of trauma, fever, and so forth it may at any time be called into activity and provoke a visible herpetic lesion.*"

Although their relationships to HSV and VZV were by no means appreciated, the more subtle members of the herpesvirus family began to be discovered after an interval of many hundreds of years. The first of these was human cytomegalovirus (HCMV), which was initially associated with human disease through the detection of enlarged cells containing unusual cytoplasmic inclusions in the urine and organs of infants who were born with signs of intrauterine damage that had been attributed to syphilis. In the early 1950s, HCMV as well as VZV were the first human herpesviruses to be isolated in cultured cells. Within a decade, Epstein-Barr virus (EBV) particles were found in Burkitt's lymphoma cells and EBV was shown to be associated with mononucleosis. By the mid-1990s, three more human herpesviruses, HHV-6A, HHV-6B and HHV-7, which share a tropism for T lymphocytes, were discovered and the etiologic agent of the unusual vascular skin tumor called 'Kaposi's sarcoma, first described in 1872, was identified as "Kaposi's sarcoma-asscoiated herpesvirus (KSHV, HHV8). These four new human herpesviruses were identified during the early years of the human immunodeficiency virus (HIV) epidemic because these viruses caused aggressive disease in HIV-infected patients or were discovered during intensive research on human T cell biology. In each instance, discovery of the human herpesviruses paralleled technologic progress, illustrated by animal models for HSV, cell culture methods for VZV and CMV, the cultivation of B lymphocytes for the detection of EBV and of T lymphocytes for identification of HHV-6 and 7, and differential nucleic acid detection for revealing the existence of HHV8.

Molecular genetics methods demonstrate that the human herpesviruses share a common ancestor. However, each virus has evolved to occupy a particular niche during millions of years of co-evolution with their primate, and eventually human, host. Understanding the nuances of the adaptive strategies that have allowed all of these viruses to be transmitted efficiently and to persist so successfully in the human population, and often in the same individual, constitutes a fascinating enterprise. At the same time, infections caused by these ubiquitous viruses create a substantial global burden of disease affecting healthy and immunocompromised patients and among people living in developed and developing countries. Because of their serious and potentially life-threatening consequences, the human herpesviruses are medically important targets for basic and clinical research.

The goal of this book is to describe the remarkable recent progress towards elucidating the basic and clinical virology of these human pathogens, in conjunction with a summary of the many new insights about their epidemiology, mechanisms of pathogenesis and immune control, approaches to clinical diagnosis and the recognition of the clinical illnesses that result from primary and recurrent herpesvirus infections across the age spectrum. All of the herpesviruses have common genes, structures, replication strategies and mechanisms of defense against the host response but each virus also has unique properties that allow it to find its particular ecological refuge. An unexpected outcome of research over the past decade is the finding that the human herpesviruses have devised many different ways to achieve the same biologic effect, as illustrated by their unique strategies for down-regulation of major histocompatibility complex proteins. Functional similarities exist among these viruses even when they do not share similar genes or infect similar tissues. Each chapter of the book explores these viral themes and variations from the virologic and clinical perspectives. The contributions of the many distinguished authors highlight the basic science aspects of the field, emphasizing the comparative virology of the human herpesviruses and virus-host cell interactions, and the significant clinical developments, including antiviral drugs and vaccines, that are essential for the best practice of medicine in the 21st century. The concluding chapter illustrates how therapies for cancer may emerge from these advances in basic and clinical research, to create a fundamentally new era in the complex history of the relationship between the human herpesviruses and their hosts.

The editors are deeply grateful for the generosity of the authors who have shared their comprehensive knowledge of the human herpesviruses. We hope that this book will serve as a resource for investigators and physicians, and most importantly, that it will motivate a new generation of students and trainees to address the many unresolved questions about these herpesviruses as agents of human disease. Since the genomes of all of these viruses have been sequenced, it is obvious that many genes exist for which functions have not been identified and we now understand that most herpesviral proteins can be expected to have multiple functions. Basic research on the human herpesviruses also reveals fundamental facts about human cellular biology, including surface receptors, metabolic pathways, cell survival mechanisms, malignant transformation as well as innate antiviral defenses. In the clinical realm, every improvement in diagnostic methods expands the spectrum of clinical disorders that are recognized as being caused by these viruses. Clinical interventions exist that could not have been imagined fifty years ago but the need for better therapeutic and preventive measures has become even more apparent as the burden of herpesvirus disease is defined with precision. Given that four human herpesviruses have been discovered in the past 15 years, are there others?

Part I

Introduction: definition and classification of the human herpesviruses

Edited by Bernard Roizman

1

Overview of classification

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Introduction

Taxonomy aims to structure relationships among diverse organisms in order to provide a broader understanding of Nature than is afforded by consideration of organisms in isolation. Since biological systems are shaped by evolution, which is not influenced by the human desire to impose order, any taxonomical scheme is bound to be incomplete and to some extent arbitrary. The criteria applied are necessarily confined to what is technically possible, and thus taxonomy has an important historical component. In addition, taxonomy develops conservatively, since striving for the ideal must be tempered by the need to maintain utility. It is also an unfortunate fact that taxonomy provides fertile soil for debate among a few but is of little interest to most. However, it is beyond dispute that the setting of herpesviruses in a taxonomical framework is vital for understanding the origins and behavior of this fascinating family of organisms.

Historically, herpesvirus taxonomy has been addressed since 1971 by the International Committee on Taxonomy of Viruses (ICTV) (Wildy, 1971). A provisional approach to endowing herpesviruses with formal names (Roizman *et al.*, 1973) was followed by grouping into subfamilies largely on the basis of biological criteria (Roizman *et al.*, 1981). This effort was rather successful, but not free from what turned out in hindsight to be a few misclassifications (Roizman *et al.*, 1992). Further division of the subfamilies into genera utilized molecular data to a greater extent than before, primarily in relation to genome characteristics such as size and structure (Roizman *et al.*, 1992). In the latest report of the ICTV Herpesvirus Study Group (Davison *et al.*, 2005), the family *Herpesviridae* consists of three subfamilies: *Alphaherpesvirinae* (containing the *Simplexvirus*, *Varicellovirus*, *Mardivirus* and *Iltovirus* genera), *Betaherpesvirinae* (containing the *Cytomegalovirus*, *Muromegalovirus* and *Roseolovirus* genera) and *Gammaherpesvirinae* (containing the *Lymphocryptovirus* and *Rhadinovirus* genera). In addition, there is a genus (*Ictalurivirus*) unattached to any subfamily and a large number of species not assigned to genera. The current list is given in Table 1.1. All but one of the viruses assigned to taxa infect mammals or birds, although a substantial number of unassigned herpesviruses have lower vertebrate (reptilian, amphibian and fish) or invertebrate (bivalve) hosts.

Morphological criteria

The primary criterion for inclusion of an agent in the family *Herpesviridae* is that of virion morphology. The virion is spherical, and comprises four major components: the core, the capsid, the tegument and the envelope (see Chapter 3). The diameter of the virion depends on the viral species, but is approximately 200 nm. The core consists of a single copy of a linear, double-stranded DNA molecule packaged at high density into the capsid. The capsid is an icosahedron, and has an external diameter of 125–130 nm. It consists of 162 capsomeres, 12 of which are pentons and 150 hexons, each containing five and six copies, respectively, of the major capsid protein. The capsomeres are joined via the triplexes, each of which contains two copies of one protein and one copy of another. The tegument, which surrounds the capsid, contains perhaps 30 or more viral protein species and is poorly defined structurally. In the tegument, structures positioned with symmetry corresponding to that of the capsid are detectable only in the region close to the capsid. The lipid envelope surrounds the exterior of the tegument, and is studded with at least ten viral membrane

Table 1.1. Herpesvirus taxonomy and nomenclature

Formal name[a]	Abbrev.	Common name[b]	Abbrev.[c]
Subfamily Alphaherpesvirinae			
Genus Simplexvirus			
Ateline herpesvirus 1	AtHV-1	Spider monkey herpesvirus	
Bovine herpesvirus 2	BoHV-2	Bovine mamillitis virus	
Cercopithecine herpesvirus 1	CeHV-1	B-virus	HVB
Cercopithecine herpesvirus 2	CeHV-2	SA8 virus	
Cercopithecine herpesvirus 16	CeHV-16	Herpesvirus papio 2	
Human herpesvirus 1	HHV-1	Herpes simplex virus [type] 1	HSV-1
Human herpesvirus 2	HHV-2	Herpes simplex virus [type] 2	HSV-2
Macropodid herpesvirus 1	MaHV-1	Parma wallaby herpesvirus	
Macropodid herpesvirus 2	MaHV-2	Dorcopsis wallaby herpesvirus	
Saimiriine herpesvirus 1	SaHV-1	Herpesvirus tamarinus	
Genus Varicellovirus			
Bovine herpesvirus 1	BoHV-1	Infectious bovine rhinotracheitis virus	BHV-1
Bovine herpesvirus 5	BoHV-5	Bovine encephalitis virus	BHV-5
Bubaline herpesvirus 1	BuHV-1	Water buffalo herpesvirus	
Canid herpesvirus 1	CaHV-1	Canine herpesvirus	
Caprine herpesvirus 1	CpHV-1	Goat herpesvirus	
Cercopithecine herpesvirus 9	CeHV-9	Simian varicella virus	SVV
Cervid herpesvirus 1	CvHV-1	Red deer herpesvirus	
Cervid herpesvirus 2	CvHV-2	Reindeer herpesvirus	
Equid herpesvirus 1	EHV-1	Equine abortion virus	
Equid herpesvirus 3	EHV-3	Equine coital exanthema virus	
Equid herpesvirus 4	EHV-4	Equine rhinopneumonitis virus	
Equid herpesvirus 8	EHV-8	Asinine herpesvirus 3	
Equid herpesvirus 9	EHV-9	Gazelle herpesvirus	
Felid herpesvirus 1	FeHV-1	Feline rhinotracheitis virus	
Human herpesvirus 3	HHV-3	Varicella-zoster virus	VZV
Phocid herpesvirus 1	PhoHV-1	Harbour seal herpesvirus	
Suid herpesvirus 1	SuHV-1	Pseudorabies virus	PRV
Tentative species in genus Varicellovirus			
Equid herpesvirus 6	EHV-6	Asinine herpesvirus 1	
Genus Mardivirus			
Gallid herpesvirus 2	GaHV-2	Marek's disease virus type 1	MDV-1
Gallid herpesvirus 3	GaHV-3	Marek's disease virus type 2	MDV-2
Meleagrid herpesvirus 1	MeHV-1	Turkey herpesvirus	HVT
Genus Iltovirus			
Gallid herpesvirus 1	GaHV-1	Infectious laryngotracheitis virus	ILTV
Unassigned species in subfamily Alphaherpesvirinae			
Psittacid herpesvirus 1	PsHV-1	Parrot herpesvirus	
Subfamily Betaherpesvirinae			
Genus Cytomegalovirus			
Cercopithecine herpesvirus 5	CeHV-5	African green monkey cytomegalovirus	SCMV
Cercopithecine herpesvirus 8	CeHV-8	Rhesus monkey cytomegalovirus	RhCMV
Human herpesvirus 5	HHV-5	Human cytomegalovirus	HCMV
Pongine herpesvirus 4	PoHV-4	Chimpanzee cytomegalovirus	CCMV
Tentative species in genus Cytomegalovirus			
Aotine herpesvirus 1	AoHV-1	Herpesvirus aotus 1	
Aotine herpesvirus 3	AoHV-3	Herpesvirus aotus 3	

Table 1.1. (*cont.*)

Formal name[a]	Abbrev.	Common name[b]	Abbrev.[c]
Genus Muromegalovirus			
Murid herpesvirus 1	MuHV-1	Mouse cytomegalovirus	MCMV
Murid herpesvirus 2	MuHV-2	Rat cytomegalovirus	RCMV
Genus Roseolovirus			
Human herpesvirus 6	HHV-6		
Human herpesvirus 7	HHV-7		
Unassigned species in subfamily Betaherpesvirinae			
Caviid herpesvirus 2	CavHV-2	Guinea pig cytomegalovirus	GPCMV
Tupaiid herpesvirus 1	TuHV-1	Tree shrew herpesvirus	THV
Subfamily Gammaherpesvirinae			
Genus Lymphocryptovirus			
Callitrichine herpesvirus 3	CalHV-3	Marmoset lymphocryptovirus	Marmoset LCV
Cercopithecine herpesvirus 12	CeHV-12	Herpesvirus papio	
Cercopithecine herpesvirus 14	CeHV-14	African green monkey EBV-like virus	
Cercopithecine herpesvirus 15	CeHV-15	Rhesus lymphocryptovirus	Rhesus LCV
Human herpesvirus 4	HHV-4	Epstein-Barr virus	EBV
Pongine herpesvirus 1	PoHV-1	Herpesvirus pan	
Pongine herpesvirus 2	PoHV-2	Orangutan herpesvirus	
Pongine herpesvirus 3	PoHV-3	Gorilla herpesvirus	
Genus Rhadinovirus			
Alcelaphine herpesvirus 1	AlHV-1	Malignant catarrhal fever virus	AHV-1
Alcelaphine herpesvirus 2	AlHV-2	Hartebeest malignant catarrhal fever virus	
Ateline herpesvirus 2	AtHV-2	Herpesvirus ateles	HVA
Bovine herpesvirus 4	BoHV-4	Movar virus	BHV-4
Cercopithecine herpesvirus 17	CeHV-17	Rhesus rhadinovirus	RRV
Equid herpesvirus 2	EHV-2		
Equid herpesvirus 5	EHV-5		
Equid herpesvirus 7	EHV-7	Asinine herpesvirus 2	
Hippotragine herpesvirus 1	HiHV-1	Roan antelope herpesvirus	
Human herpesvirus 8	HHV-8	Kaposi's sarcoma-associated herpesvirus	KSHV
Murid herpesvirus 4	MuHV-4	Murine gammaherpesvirus 68	MHV-68
Mustelid herpesvirus 1	MusHV-1	Badger herpesvirus	
Ovine herpesvirus 2	OvHV-2	Sheep-associated malignant catarrhal fever virus	
Saimiriine herpesvirus 2	SaHV-2	Herpesvirus saimiri	HVS
Tentative species in genus Rhadinovirus			
Leporid herpesvirus 1	LeHV-1	Cottontail rabbit herpesvirus	
Leporid herpesvirus 2	LeHV-2	Herpesvirus cuniculi	
Leporid herpesvirus 3	LeHV-3	Herpesvirus sylvilagus	
Marmodid herpesvirus 1	MarHV-1	Woodchuck herpesvirus	
Unassigned species in subfamily Gammaherpesvirinae			
Callitrichine herpesvirus 1	CalHV-1	Herpesvirus saguinus	
Unassigned genus Ictalurivirus* in family *Herpesviridae			
Ictalurid herpesvirus 1	IcHV-1	Channel catfish virus	CCV
Unassigned viruses in family Herpesviridae			
Acipenserid herpesvirus 1	AciHV-1	White sturgeon herpesvirus 1	
Acipenserid herpesvirus 2	AciHV-2	White sturgeon herpesvirus 2	
Acciptrid herpesvirus 1	AcHV-1	Bald eagle herpesvirus	
Anatid herpesvirus 1	AnHV-1	Duck plague herpesvirus	
Anguillid herpesvirus 1	AngHV-1	Japanese eel herpesvirus	

(*cont.*)

Table 1.1. (*cont.*)

Formal name[a]	Abbrev.	Common name[b]	Abbrev.[c]
Ateline herpesvirus 3	AtHV-3	Herpesvirus ateles strain 73	
Boid herpesvirus 1	BoiHV-1	Boa herpesvirus	
Callitrichine herpesvirus 2	CalHV-2	Marmoset cytomegalovirus	
Caviid herpesvirus 1	CavHV-1	Guinea pig herpesvirus	
Caviid herpesvirus 3	CavHV-3	Guinea pig herpesvirus 3	
Cebine herpesvirus 1	CbHV-1	Capuchin herpesvirus AL-5	
Cebine herpesvirus 2	CbHV-2	Capuchin herpesvirus AP-18	
Cercopithecine herpesvirus 3	CeHV-3	SA6 virus	
Cercopithecine herpesvirus 4	CeHV-4	SA15 virus	
Cercopithecine herpesvirus 10	CeHV-10	Rhesus leukocyte-associated herpesvirus strain 1	
Cercopithecine herpesvirus 13	CeHV-13	Herpesvirus cyclopis	
Chelonid herpesvirus 1	ChHV-1	Grey patch disease of turtles	
Chelonid herpesvirus 2	ChHV-2	Pacific pond turtle herpesvirus	
Chelonid herpesvirus 3	ChHV-3	Painted turtle herpesvirus	
Chelonid herpesvirus 4	ChHV-4	Argentine turtle herpesvirus	
Ciconiid herpesvirus 1	CiHV-1	Black stork herpesvirus	
Columbid herpesvirus 1	CoHV-1	Pigeon herpesvirus	
Cricetid herpesvirus	CrHV-1	Hamster herpesvirus	
Cyprinid herpesvirus 1	CyHV-1	Carp pox herpesvirus	
Cyprinid herpesvirus 2	CyHV-2	Goldfish herpesvirus	
Elapid herpesvirus 1	EpHV-1	Indian cobra herpesvirus	
Elephantid herpesvirus 1	ElHV-1	Elephant [loxodontal] herpesvirus	
Erinaceid herpesvirus 1	ErHV-1	European hedgehog herpesvirus	
Esocid herpesvirus 1	EsHV-1	Northern pike herpesvirus	
Falconid herpesvirus 1	FaHV-1	Falcon inclusion body diseases	
Gruid herpesvirus 1	GrHV-1	Crane herpesvirus	
Iguanid herpesvirus 1	IgHV-1	Green iguana herpesvirus	
Lacertid herpesvirus	LaHV-1	Green lizard herpesvirus	
Lorisine herpesvirus 1	LoHV-1	Kinkajou herpesvirus	
Murid herpesvirus 3	MuHV-3	Mouse thymic herpesvirus	
Murid herpesvirus 5	MuHV-5	Field mouse herpesvirus	
Murid herpesvirus 6	MuHV-6	Sand rat nuclear inclusion agents	
Ostreid herpesvirus 1	OsHV-1	Pacific oyster herpesvirus	OHV
Ovine herpesvirus 1	OvHV-1	Sheep pulmonary adenomatosis-associated herpesvirus	
Percid herpesvirus 1	PeHV-1	Walleye epidermal hyperplasia	
Perdicid herpesvirus 1	PdHV-1	Bobwhite quail herpesvirus	
Phalacrocoracid herpesvirus 1	PhHV-1	Cormorant herpesvirus	
Pleuronectid herpesvirus 1	PlHV-1	Turbot herpesvirus	
Ranid herpesvirus 1	RaHV-1	Lucké frog herpesvirus	
Ranid herpesvirus 2	RaHV-2	Frog herpesvirus 4	
Salmonid herpesvirus 1	SalHV-1	Herpesvirus salmonis	
Salmonid herpesvirus 2	SalHV-2	*Oncorhynchus masou* herpesvirus	
Sciurid herpesvirus 1	ScHV-2	European ground squirrel cytomegalovirus	
Sciurid herpesvirus 2	ScHV-2	American ground squirrel cytomegalovirus	
Sphenicid herpesvirus 1	SpHV-1	Black footed penguin herpesvirus	
Strigid herpesvirus 1	StHV-1	Owl hepatosplenitis herpesvirus	
Suid herpesvirus 2	SuHV-2	Swine cytomegalovirus	

[a] Type species of genera are in italics.
[b] Some viruses have several common names. Only one is given for each.
[c] The list is restricted to abbreviations used in this publication.
Adapted from Davison *et al.* (2005).

glycoproteins, in addition to some cellular proteins. The protein composition of the tegument and envelope varies widely across the family.

Serological criteria

In contrast to virion morphology, which operates as a criterion at the level of the family, serological relationships are useful only for detecting closely related viruses. Neutralizing antibodies form a subset of serological tools, and are directed against some of the envelope glycoproteins.

Biological criteria

The observation that several distinct herpesviruses have been found in the most extensively studied animals implies that the number of herpesvirus species in Nature must far exceed that catalogued to date. The natural host range of individual viruses is usually restricted to a single species. Occasional transfer to other species can occur, although it could be argued that the settings involved (farms, zoos and keeping pets) are the results of human activities. In experimental animal systems, some members of the *Alphaherpesvirinae* can infect a wide variety of species, whereas *Beta-* and *Gammaherpesvirinae* are very restricted. The same general observation characterizes growth in cell culture.

Herpesviruses are highly adapted to their hosts, and severe symptoms of infection are usually limited to very young or immunosuppressed individuals. Natural transmission routes range from aerosol spread to mucosal contact. Most herpesviruses establish a systemic infection associated with a cell-associated viraemia, although infection with some members of genus *Simplexvirus* is limited to the epithelium at the inoculation site and to innervating sensory neurons. Herpesviruses have elaborate means of modulating the host responses to infection, and are able to establish lifelong latent infections. In simplified, general terms, the cell types involved in latency are the neuron for the *Alphaherpesvirinae*, the monocyte lineage for the *Betaherpesvirinae*, and lymphocytes for the *Gammaherpesvirinae*.

Genomic criteria

Herpesvirus genomes studied to date range in size from about 125 to 240 kbp, and the most extensively characterized contain from about 70 to 165 genes. Prior to the generation of extensive sequence data, genome structures (see Chapter 2) were an aid to classification. However, the usefulness of this criterion is limited, since similar structures have evidently evolved more than once in the family. Nucleic acid hybridization data also provided input and, like serological data, are limited to demonstrating relationships between closely related viruses. As with other groups of organisms, data derived from nucleotide and amino acid sequences have gained increasing prominence and now dominate herpesvirus taxonomy. Figure 1.1 shows one example of such data, a phylogenetic tree based upon amino acid sequence alignments (McGeoch *et al.*, 1994, 1995, 2000). Another approach yielded different schemes of relationships, but was based on analytical criteria not widely accepted in depicting evolutionary relationships (Karlin *et al.*, 1994).

It has long been thought from the apparent adaptation of herpesviruses to their hosts that a substantial degree of co-evolution has occurred. Similarities between the phylogenetic relationships among the viruses and those among their hosts provide strong support for this model, and in some instances indicate that co-speciation has occurred. A number of possible exceptions have been noted and are discussed in further detail in Chapter 2.

Species definition

A virus species is defined as a polythetic class of viruses constituting a replicating lineage and occupying a particular ecological niche (Van Regenmortel, 1989, 1990). Members of a polythetic class share a subset of properties, with each property possessed by several members but no property possessed by all. Herpesviruses are defined as separate species if their nucleotide sequences differ in a readily assayable and distinctive manner across the entire genome and if they occupy different ecological niches by virtue of their distinct epidemiology and pathogenesis or their distinct natural hosts (Roizman *et al.*, 1992; Roizman and Pellett, 2001; Davison *et al.*, 2005). However, genomic data have come to dominate biological properties, with taxa corresponding to genetic lineages defined by sequence comparisons and identification of genes unique to certain lineages. An increasing number of herpesviruses in the tissues of various animals are being inferred from short PCR-derived sequences, usually from a single locus in the genome and often in the absence of any other information. These "virtual viruses" cannot readily be classified under the current species definition. However, their incorporation (perhaps in a special category) could be facilitated by

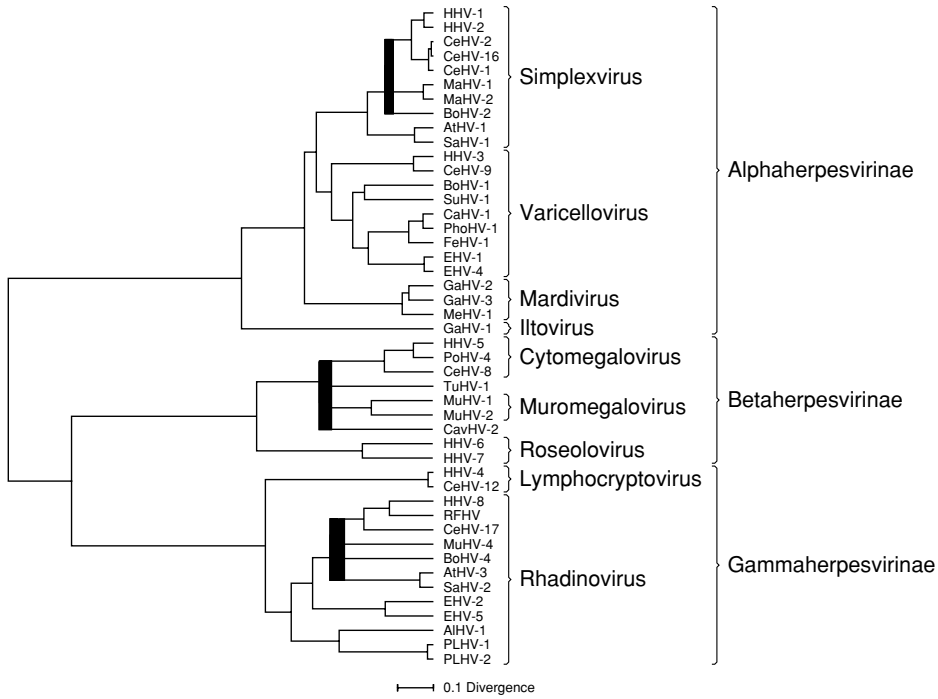

Fig. 1.1. Composite phylogenetic tree for herpesviruses. The tree is based on amino acid sequence alignments of eight sets of homologous genes, constructed from maximum-likelihood trees for subsets of these genes, with molecular clock imposed. Thick lines designate regions of uncertain branching. Formal species abbreviations and designations for genera and subfamilies are given on the right (see Table 1.1). Viruses that are not yet incorporated formally into genera are denoted in italics. Three unclassified viruses are included (RFHV, retroperitoneal fibromatosis herpesvirus of macaques; PLHV-1 and PLHV-2, porcine lymphotropic herpesviruses 1 and 2). Modified from McGeoch *et al.* (2000) with permission from the American Society for Microbiology.

addition to the species definition of a third criterion, that of phylogeny based on the relatedness of conserved genes. Recognition that taxonomy should reflect evolutionary history would also aid rational incorporation of herpesviruses of lower vertebrates and invertebrates into a taxonomy that is currently dominated by herpesviruses of higher vertebrates. Current problems in this area, and a suggested solution, are given in Chapter 2.

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2

Comparative analysis of the genomes

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Introduction

Members of the family *Herpesviridae* replicate their genomes in the infected cell nucleus and have a characteristic virion morphology, which consists of the envelope, tegument, capsid and core (Davison and Clements, 1997). An extensive description of virion structure is given in Chapter 3. The present chapter focuses on the viral genome, which occupies the core of the virus particle. Electron microscopy of negatively stained capsids gives the impression that the core consists of the viral DNA molecule wrapped toroidally around a protein spindle (Furlong *et al.*, 1972). Images reconstructed from electron micrographs of virions frozen in ice in the absence of stain, a technique by which morphology is better preserved, show that the core consists of the DNA packed at high density in liquid crystalline form, probably as a spool lacking a spindle (Booy *et al.*, 1991; Zhou *et al.*, 1999).

Herpesvirus genomes consist of linear, double-stranded DNA molecules that range in size from about 125 to 240 kbp and in nucleotide composition from 32 to 75% G+C, depending on the virus species (Honess, 1984). The genome termini are not covalently closed (as in the *Poxviridae;* Moss, 2001) or covalently linked to a protein (as in the *Adenoviridae*; Shenk, 2001). In those herpesvirus genomes that have been examined in sufficient detail, unpaired nucleotides are present at the termini; for example, HSV-1, VZV and HCMV have a single 3′-overhanging nucleotide at each terminus (Mocarski and Roizman, 1982; Davison, 1984; Tamashiro and Spector, 1986). Larger herpesvirus genomes are accommodated in larger capsids, but the relationship is not proportional, as the packing density of the DNA varies somewhat between species (Trus *et al.*, 1999; Bhella *et al.*, 2000). The reasons for the striking range in nucleotide composition of herpesvirus genomes are not clear, but a similar phenomenon is found in other virus families and in cellular organisms. In contrast to the *Alpha-* and *Betaherpesvirinae*, the genomes of most *Gammaherpesvirinae* are generally deficient in the CG dinucleotide (Table 2.1). In vertebrate genomes, this phenomenon is thought to be due to spontaneous deamination of 5-methylcytosine residues in DNA to thymidine residues, followed by fixation through DNA replication. CG depletion in herpesviruses, and concomitant enrichment in TG and CA, has been taken as indicative of latency in dividing cell populations, in which the latent genome is obliged to replicate as host cells divide (Honess *et al.*, 1989). Thus, HSV-1, which is resident in non-dividing neurons, has a CG content consistent with its nucleotide composition, whereas EBV, which latently infects dividing B cell populations, is depleted. Local CG suppression of the major immediate early gene locus of HCMV has also been noted (Honess *et al.*, 1989).

Genome structures

Herpesvirus genomes are not simple lengths of unique DNA, but characteristically contain direct or inverted repeats. The reasons for this are not known, but it is intriguing that similar structures appear to have arisen independently on several occasions during herpesvirus evolution. Herpesvirus genomes are thought to replicate by circularization, followed by production of concatemers and cleavage of unit-length genomes during packaging into capsids (Boehmer and Lehman, 1997). The explanation for the presence of repeats is probably connected in some way with the mode of DNA replication, rather than with any advantage gained by having multiple copies of certain genes. Although greater expression would be a consequence of repeated genes, this appears a simplistic explanation in an evolutionary context, since subtler processes of nucleotide

Table 2.1. Sequenced herpesvirus genomes

Common name	Strain[a]	Abbreviation		Accession	Size (bp)[b]	Composition[c]		Reference
		Common	Formal			G+C	CG	
Mammalian herpesvirus group[d]								
Alphaherpesvirinae								
Simplexvirus								
Herpes simplex virus type 1	17	HSV-1	HHV-1	X14112	152261	68.3	1.01	McGeoch *et al.* (1988)
Herpes simplex virus type 2	HG52	HSV-2	HHV-2	Z86099	154746	70.4	1.06	Dolan *et al.* (1998)
B virus	E2490	HVB	CeHV-1	AF533768	156789	74.5	1.09	Perelygina *et al.* (2003)
SA8	B264	SA8	CeHV-2	AY714813	150715	76.0	1.09	Tyler *et al.* (2005)
Herpes papio 2	X313	HVP2	CeHV-16	DQ149153	156487	76.1	1.08	Tyler and Severini (2006)
Varicellovirus								
Varicella-zoster virus	Dumas	VZV	HHV-3	X04370	124884	46.0	1.14	Davison and Scott (1986)
	Oka vaccine			AB097932	125078			Gomi *et al.* (2002)
	Oka parental			AB097933	125125			Gomi *et al.* (2002)
	MSP			AY548170	124883			Grose *et al.* (2004)
	BC			AY548171	125459			Grose *et al.* (2004)
	Varilrix			DQ008354	124821			Vassilev (2005)
	Varilrix			DQ008355	124815			Vassilev (2005)
	HJO			AJ871403	124928			Fickenscher *et al.* (unpublished)
Simian varicella virus	Delta	SVV	CeHV-9	AF275348	124138	40.4	1.12	Gray *et al.* (2001)
Bovine herpesvirus 1	[Cooper]	BHV-1	BoHV-1	AJ004801	135301	72.4	1.19	Schwyzer & Ackermann (1996)
Bovine herpesvirus 5	SSV507/99	BHV-5	BoHV-5	AY261359	138390	74.9	1.17	Delhon *et al.* (2003)
Pseudorabies virus	[Kaplan]	PRV	SuHV-1	BK001744	143461	73.6	1.12	Klupp *et al.* (2004)
Equine herpesvirus 1	Ab4	EHV-1	EHV-1	AY665713	150224	56.7	0.99	Telford *et al.* (1992)
	V592			AY464052	149430			Nugent *et al.* (2006)
Equine herpesvirus 4	NS80567	EHV-4	EHV-4	AF030027	145597	50.5	0.93	Telford *et al.* (1998)
Mardivirus								
Marek's disease virus type 1	Md5	MDV-1	GaHV-2	AF243438	177874	44.1	1.01	Tulman *et al.* (2000)
	GA			AF147806	174077			Lee *et al.* (2000)
	Md11			[AY510475]	170950			Niikura *et al.* (unpublished)
Marek's disease virus type 2	HPRS24	MDV-2	GaHV-3	AB049735	164270	53.6	1.23	Izumiya *et al.* (2001)
Turkey herpesvirus	FC126	HVT	MeHV-1	AF291866	159160	47.6	1.11	Afonso *et al.* (2001)
Iltovirus								
Infections laryngotracheitis virus	[SA-2]	ILTV	GaHV-1	NC006623	148687	48.2	1.01	Thureen and Keeler (2006)
Psittacid herpesvirus 1[e]	97-0001	PsHV-1	PsHV-1	AY372243	163025	60.9	1.21	Thureen and Keeler (2006)
Betaherpesvirinae								
Cytomegalovirus								
Human cytomegalovirus	Merlin	HCMV	HHV-5	AY446894	235645	57.5	1.19	Dolan *et al.* (2004)
	AD169			X17403	229354			Chee *et al.* (1990)
	AD169			BK000394	230287			Davison *et al.*(2003a)
	AD169			[AC146999]	[233739]			Murphy *et al.* (2003b)
	Towne			[AY315197]	[231236]			Dunn *et al.* (2003)
	Towne			[AC146851]	[229483]			Murphy *et al.* (2003b)
	Toledo			[AC146905]	[226889]			Murphy *et al.* (2003b)
	PH			[AC146904]	[229700]			Murphy *et al.* (2003b)
	TR			[AC146906]	[234881]			Murphy *et al.* (2003b)
	FIX			[AC146907]	[229209]			Murphy *et al.* (2003b)
Chimpanzee cytomegalovirus	–	CCMV	PoHV-4	AF480884	241087	61.7	1.11	Davison *et al.* (2003a)
Rhesus cytomegalovirus	68-1	RhCMV	CeHV-8	AY186194	221454	49.1	0.99	Hansen *et al.* (2003)
Unassigned								
Tupaiid herpesvirus	2	THV	TuHV-1	AF281817	195859	66.6	1.28	Bahr & Darai (2001)
Muromegalovirus								
Murine cytomegalovirus	Smith	MCMV	MuHV-1	U68299	230278	58.7	1.22	Rawlinson *et al.* (1996)
Rat cytomegalovirus	Maastricht	RCMV	MuHV-2	AF232689	230138	61.0	1.25	Vink *et al.* (2000)
Roseolovirus								
Human herpesvirus 6	U1102	HHV-6A	HHV-6	X83413	159321	42.4	1.13	Gompels *et al.* (1995)
	Z29	HHV-6B		AF157706	162114	42.8	1.10	Dominguez *et al.* (1999)
	HST			AB021506	161573			Isegawa *et al.* (1999)

(*cont.*)

Table 2.1. (*cont.*)

Common name	Strain[a]	Abbreviation		Accession	Size (bp)[b]	Composition[c]		Reference
		Common	Formal			G+C	CG	
Human herpesvirus 7	JI	HHV-7	HHV-7	U43400	144861	35.3	0.80	Nicholas (1996)
	RK			AF037218	153080			Megaw *et al.* (1998)
Gammaherpesvirinae								
Lymphocryptovirus								
Epstein–Barr virus	[B95-8]	EBV	HHV-4	AJ507799	171823	59.5	0.61	de Jesus *et al.* (2003)
	GD1			AY961628	171656			Zeng *et al.* (2005)
	AG876			DQ279927	172764			Dolan *et al.* (2006)
	B95-8			V01555	172281			Baer *et al.* (1984)
Marmoset lymphocryptovirus	CJ0149	marmoset LCV	CalHV-3	AF319782	149696	49.3	0.70	Rivailler *et al.* (2002a)
Rhesus lymphocryptovirus	LCL8664	rhesus LCV	CeHV-15	AY037858	171096	61.9	0.68	Rivailler *et al.* (2002b)
Rhadinovirus								
Human herpesvirus 8	BC-1	HHV-8	HHV-8	U75698	[137508]	53.5	0.81	
				U75699	801	84.5	0.92	Russo *et al.* (1996)
	–			U93872	[133661]			Neipel *et al.* (1997)
Rhesus rhadinovirus	17577	RRV	CeHV-17	AF083501	133719	52.2	1.11	Searles *et al.* (1999)
	26-95			AF210726	130733			Alexander *et al.* (2000)
Murine herpesvirus 68[f]	WUMS	MHV-68	MuHV-4	U97553	119450	47.2	0.43	Virgin *et al.* (1997)
	g2.4			AF105037	119550			Nash *et al.* (2001)
Bovine herpesvirus 4	66-p-347	BHV-4	BoHV-4	AF318573	108873	41.4	0.23	Zimmermann *et al.* (2001)
				AF092919	2267	71.2	0.42	
Herpesvirus ateles	73	HVA	AtHV-3	AF083424	108409	36.6	0.40	Albrecht (2000)
				AF126541	1582	77.1	0.79	
Herpesvirus saimiri	A11	HVS	SaHV-2	X64346	112930	34.5	0.33	Albrecht *et al.* (1992)
				K03361	1444	70.9	0.61	
	C488			AJ410493	113027			Ensser *et al.* (2003)
				AJ410494	1458			
Equine herpesvirus 2	86/67	EHV-2	EHV-2	U20824	184427	57.5	0.63	Telford *et al.* (1995)
Alcelaphine herpesvirus 1	C500	AHV-1	AlHV-1	AF005370	130608	46.2	0.42	Ensser *et al.* (1997)
				AF005368	1113	72.0	0.69	
Ovine herpesvirus 2	BJ1035	OvHV-2	OvHV-2	AY839756	135135	52.9	0.58	Stewart *et al.* (unpublished)
Fish herpesvirus group[d]								
Undefined subfamily								
Ictalurivirus								
Channel catfish virus	Auburn 1	CCV	IcHV-1	M75136	134226	56.2	1.1	Davison (1992)
Bivalve herpesvirus group[d]								
Undefined subfamily								
Undefined genus								
Ostreid herpesvirus 1	–	OHV	OsHV-1	AY509253	207439	38.7	0.68	Davison *et al.* (2005)

[a] Square brackets indicate the strain that was used most extensively in assembling a sequence combining data from several strains. A hyphen indicates that the strain was not specified.

[b] Sizes were obtained from the latest version of the accessions, and may differ from those in the references through correction of errors. Square brackets indicate sequences that fall marginally short of full length: for MDV-1 and HCMV strains, sequences are for bacterial artificial chromosomes, the sizes representing a deleted form of the genome plus the vector; for HHV-8 strains, the sequence at the right end of the unique region was not determined. For members of subfamily *Gammaherpesvirinae* other than EHV-2, actual genome sizes are larger than those listed (approximately 150–180 kbp), owing to the presence of variable copy numbers of terminal repeats at both ends of the genome. Where a single value is given, this represents either the unique region flanked by partial terminal repeats or the unique region only. Where two values are given, the first is for the unique region and the second is for the terminal repeat.

[c] G + C content is given as moles %, CG content is given as observed/expected frequency, taking into account overall nucleotide composition. Where multiple strains have been sequenced for a species, values are given for one strain only. For members of the *Gammaherpesvirinae*, values are given for the deposited sequences, which consist either of the unique region flanked by partial terminal repeat sequence, the unique region only, or separate accessions for the unique region and terminal repeat.

[d] This taxon is used for the purposes of discussion, and, unlike the others in the Table, has no formal standing.

[e] PsHV-1 is the closest relative of ILTV and is placed informally in this genus.

[f] MHV-68 strains g2.4 and WUMS are essentially identical, since the latter was derived from the former.

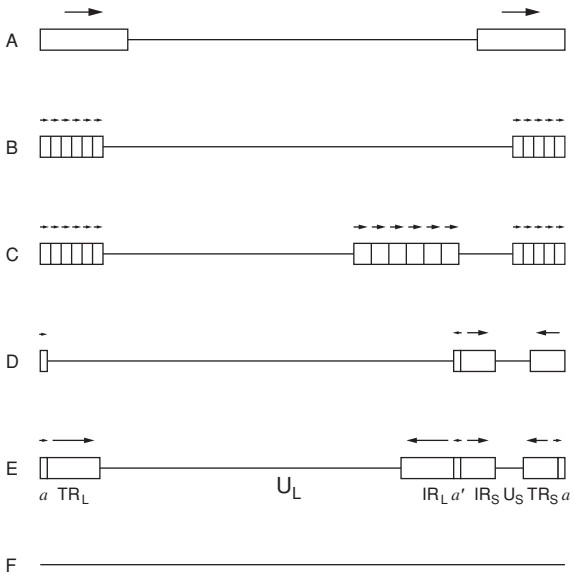

Fig. 2.1. Classes of herpesvirus genome structures (not to scale) as defined by Roizman and Pellett (2001). Unique and repeat regions are shown as horizontal lines and rectangles, respectively. The orientations of repeats are shown by arrows. The nomenclature of unique and repeat regions, including the terminal redundancy (*a*) and its internal, inverted copy (*a*′), is indicated for the class E genome.

substitution can readily alter transcriptional levels over a much greater range. In addition, repeats often do not contain protein-coding regions. As elaborated below, certain genomes exhibit a further structural complexity known as segment inversion, in which unique regions flanked by inverted repeats are found in both orientations in virion DNA. Thus, a genome with two such unique regions would produce either two or four isomers depending on whether one or both regions invert. This phenomenon is probably a consequence of recombination between repeats in concatemeric DNA. Isomers are functionally equivalent (Jenkins and Roizman, 1986), and segment inversion appears to be unrelated to the biology of the virus.

Figure 2.1 shows the major classes of genome structure found among the herpesviruses, as summarized by Roizman and Knipe (2001). The class A genome consists of a unique sequence flanked by a direct repeat. It was first described for CCV (Chousterman *et al.*, 1979), but is also represented among the *Betaherpesvirinae* (HHV-6: Lindquester and Pellett, 1991; Martin *et al.*, 1991; HHV-7: Dominguez *et al.*, 1996; Ruvolo *et al.*, 1996) and one member of the *Gammaherpesvirinae* (EHV-2; Browning and Studdert, 1989). In these examples, the direct repeat is several kbp in size. Other members of the *Betaherpesvirinae* also have this arrangement, but the repeat is smaller, at 504 bp in RCMV (Vink *et al.*, 1996) and 30–31 bp in MCMV (Marks and Spector, 1988; Rawlinson *et al.*, 1996).

Class B genomes also have directly repeated sequences at the termini, but these consist of variable copy numbers of a tandemly repeated sequence of 0.8–2.3 kbp. This arrangement characterizes most *Gammaherpesvirinae* in the *Rhadinovirus* genus, such as HVS and HHV-8 (Bornkamm *et al.*, 1976; Russo *et al.*, 1996). The repeated regions may comprise up to 30% of the DNA molecule (Bornkamm *et al.*, 1976; Lagunoff and Ganem, 1997). The presence of additional terminal repeat sequences in inverse orientation internally in the genome gives rise to a related structure, which is present in another member of the *Gammaherpesvirinae*, cottontail rabbit herpesvirus (Cebrian *et al.*, 1989). The virion DNA of this virus exhibits segment inversion because the two unique regions are flanked by inverted repeats. The class C structure represents another derivative of class B, in which an internal set of direct repeats is present but is unrelated to the terminal set. EBV, a member of the *Gammaherpesvirinae* in the *Lymphocryptovirus* genus, has this arrangement (Given and Kieff, 1979). Segment inversion does not occur because the internal and terminal repeats are not related.

Class D genomes contain two unique regions (U_L and U_S), each flanked by inverted repeats (TR_L/IR_L and TR_S/IR_S). This structure is characteristic of *Alphaherpesvirinae* in the *Varicellovirus* genus, such as PRV and VZV (Rixon and Ben-Porat, 1979; Dumas *et al.*, 1981), and has also evolved separately in salmonid herpesvirus 1 (Davison, 1998). Segment inversion occurs inasmuch as equimolar amounts of genomes containing the two orientations of U_S are found in virion DNA, but U_L is present predominantly or completely in one orientation. The latter feature cannot be explained solely by recombination, and is probably due also to the presence of the cleavage signal solely or largely in the region comprising TR_L/IR_L and one end of U_L (Davison, 1984; Rall *et al.*, 1991).

Class E is the most complex genome structure, and was the first to be described, for HSV-1 (Sheldrick and Berthelot, 1975). It is similar to class D, except that TR_L/IR_L is much larger and segment inversion gives rise to four equimolar genome isomers (Wadsworth *et al.*, 1975; Hayward *et al.*, 1975; Delius and Clements, 1976; Clements *et al.*, 1976; Wilkie and Cortini, 1976). Also, class E genomes are terminally redundant, containing a sequence of a few hundred bp (termed the *a* sequence) that is repeated directly at the genome termini and inversely at the IR_L-IR_S junction (Sheldrick and Berthelot, 1975; Grafstrom *et al.*, 1975a,b; Wadsworth *et al.*, 1976; Hyman *et al.*, 1976).

Minor proportions of genomes contain multiple copies of the *a* sequence at the left terminus or the IR_L-IR_S junction (Wilkie, 1976; Wagner and Summers, 1978; Locker and Frenkel, 1979). The class E arrangement is characteristic of *Alphaherpesvirinae* in the *Simplexvirus* genus, and has evolved independently in the lineage giving rise to HCMV and CCMV, members of the *Betaherpesvirinae* (Weststrate *et al.*, 1980; DeMarchi, 1981; Davison *et al.*, 2003a). A structure similar to both class D and E genomes has also evolved in an invertebrate herpesvirus, OsHV-1 (Davison *et al.*, 2005). This contains two segments, each consisting of a unique region flanked by an substantial inverted repeat, linked via an additional small, non-inverting unique region. As in class E genomes, the two segments undergo inversion, but, like class D, the genome is not terminally redundant.

Class F is represented by a member of the *Betaherpesvirinae*, THV, which apparently lacks the types of inverted and direct repeats that characterize other herpesvirus genomes (Koch *et al.*, 1985; Albrecht *et al.*, 1985). However, since the genome ends of THV have not been analyzed directly, the existence of this unusual structure is considered tentative.

Genome sequences

Table 2.1 lists the 39 herpesvirus species for which genome sequences are currently available in the public databases. Additional strains have been sequenced for some species, yielding a total of 63 sequenced strains. The ease of generating data will continue to expand the number of herpesvirus species and strains sequenced in coming years. Indeed, substantial inroads have been made into large-scale studies of strain variation for certain of the human herpesviruses. It appears that the scale and extent of variation is lineage dependent, with *Betaherpesvirinae* more variable than *Gammaherpesvirinae*, and *Alphaherpesvirinae* the least variable (Murphy *et al.*, 2003b; Dolan *et al.*, 2004; Poole *et al.*, 1999; Midgley *et al.*, 2003; Muir *et al.*, 2002; Gomi *et al.*, 2002). The development of tools to study variation in increasing detail will enhance understanding of viral epidemiology, in terms both of its relation to human evolution and migration and of the changes that are occurring in human populations at the present time.

Gene content

Sequencing herpesvirus genomes is now routine, but the process of describing gene content (annotation) is not trivial. Thus, as with other groups of organisms, the quality of annotation of herpesvirus entries in the public databases varies widely. It is an unfortunate fact that no set of objective criteria is sufficient to interpret the gene content of a sequence completely. Although most genes can be catalogued relatively easily, there are genuine difficulties in identifying all of them, even in the best characterized herpesviruses.

A primary criterion in defining gene content involves identifying open reading frames (ORFs), usually those initiated by methionine (ATG) codons. A tendency to include ORFs that do not encode proteins may be reduced by setting a minimum size. Comparative genomics, which operates on the principle that genes are conserved in evolution, and algorithms that compare sequence patterns within ORFs to the protein-coding regions of known genes, are also useful. However, these tools yield results with least confidence when applied to small, spliced, overlapping or poorly conserved ORFs, and in instances where translation initiates from internal codons, alternative splicing occurs, or esoteric translational mechanisms are employed (e.g., suppression of termination codons and forms of translational editing). In addition to sequence analysis, experimental data on production of an RNA or protein from an ORF provides important imput, although even this falls short of proving functionality. Also, most approaches are aimed at identifying protein-coding genes, and cannot detect genes that encode functional transcripts that are not mRNAs.

The use of different criteria for gene identification may create a degree of uncertainty and debate, and lead to different pictures of gene layout. The case of HCMV provides a contemporary example. In the first analysis of the gene content of HCMV strain AD169, Chee *et al.* (1990) catalogued 189 protein-coding ORFs (counting duplicates once only). Later, the gene number was reduced to 147 by comparing the HCMV and CCMV genomes, allowing, where appropriate, for the presence of genes unique to either genome (Davison *et al.*, 2003a,b). As modified criteria were applied, this number rebounded in a series of increments, first to 157 (Yu *et al.*, 2003), next to 171 (Murphy *et al.*, 2003a), then to 220 (Murphy *et al.*, 2003b) and finally to 232 (Varnum *et al.*, 2004). Although the conservative numbers in this example are more supportable, the existence of unrecognized genes should not be ruled out even in well-characterized genomes, and candidates should be examined rigorously. For example, new genes were identified in previously analysed sequences for VZV (Kemble *et al.*, 2000) and HHV-8 (Glenn *et al.*, 1999).

The genes of HSV-1, presumably like those of all herpesviruses, are transcribed by host RNA polymerase II

(Wagner, 1985; Roizman and Knipe, 2001). Transcription of the first genes to be expressed, the immediate early genes, does not require ongoing protein synthesis, and is enhanced by a tegument protein at low multiplicities of infection (O'Hare, 1993). Some of the immediate early proteins regulate expression of early and late genes (Honess and Roizman, 1974). Early genes, defined as those expressed in the presence of immediate early proteins and before the onset of DNA replication, include enzymes involved in nucleotide metabolism and DNA replication and a number of envelope glycoproteins. Some late genes are expressed at low levels under early conditions, but full expression of "leaky" and "true" late genes is dependent on DNA replication; these genes encode mainly virion proteins. Although the details differ, a similar pattern of regulated gene expression is characteristic of all herpesviruses examined; for example, HCMV (Stinski, 1978), HHV-8 (Sarid *et al.*, 1998) and CCV (Silverstein *et al.*, 1995). In addition, herpesviruses express RNAs whose functions apparently do not involve translation. The best characterized are small RNAs probably transcribed by RNA polymerase III in *Gammaherpesvirinae* such as EBV (Rosa *et al.*, 1981) and MHV-68 (Bowden *et al.*, 1997). Larger noncoding RNAs transcribed by RNA polymerase II include the latency-associated transcripts in HSV-1 (Stevens *et al.*, 1987) and several virion-associated RNAs in HCMV (Bresnahan and Shenk, 2000).

With the exception of a small number of genes that are expressed by splicing from a common 5′-leader, such as the EBNA genes of EBV (Bodescot *et al.*, 1987) and the IE1 and IE2 genes of HCMV (Stenberg *et al.*, 1985), herpesvirus genes have individual promoters. However, it is common for genes to share a polyadenylation site, leading to families of 3′-coterminal transcripts (Wagner, 1985). Apart from families of duplicated genes, there is no pronounced clustering of genes on the basis of function or kinetics of expression. Splicing is uncommon throughout the family, affecting no more than about 20% of the gene number in any genome. Most splicing involves genes that are relatively recent evolutionary developments, and *Beta-* and *Gammaherpesvirinae* have more spliced genes than *Alphaherpesvirinae*.

Genome comparisons and evolution

The availability of extensive sequence data for herpesviruses has facilitated detailed phylogenetic analyses of the family based on amino acid sequence comparisons of conserved genes, as described in Chapter 1. In this section, an overview is given of genetic relatedness at selected levels in the phylogenetic tree, starting with the three major groups that encompass all known herpesviruses, proceeding to the best characterized of these groups, and ending with one subfamily in this group. In chronological terms, this proceeds from earlier to more recent evolutionary events. Detailed information on the gene content of, and the relationships between, the human herpesviruses is available elsewhere in this book.

Three major groups

Three major groups of viruses possess the herpesvirus morphology, including closely similar capsid structures, but share very little genetic similarity (Davison, 1992; Booy *et al.*, 1996; Davison *et al.*, 2005). Viruses in the best characterized group infect mammals, birds and reptiles, viruses in the second group infect amphibians and fish, and the third group contains the single known herpesvirus of an invertebrate, the oyster. Currently, the family *Herpesviridae* comprises the first group classified into three subfamilies and component genera, one member (CCV) of the second group representing an unassigned genus, and the oyster virus is a floating species. The most logical means of accommodating all known herpesviruses taxonomically would be to establish three families under the umbrella of a new order (*Herpesvirales*), containing herpesviruses of mammals, birds and reptiles, of amphibians and fish, and of bivalves, respectively. Since these taxa are presently a proposal and lack any formal standing, the terms mammalian, fish and bivalve herpesvirus groups are used to denote the proposed families in the following discussion.

Only three genes have clear counterparts in all three groups that are detectable by amino acid sequence comparisons. The proteins encoded by two (DNA polymerase and dUTPase) have ubiquitous cellular relatives and could have been captured independently from the host repertoire. The third gene apparently lacks a counterpart in the host cell but has distant relatives in T4 and similar bacteriophages (Davison, 1992; Mitchell *et al.*, 2002). The T4 gene is known to encode the ATPase subunit of a DNA packaging enzyme complex called the terminase (Rao and Black, 1988; Bhattacharyya and Rao, 1993), and the HSV-1 gene has properties that are consistent with a similar function (Yu and Weller, 1998).

The existence of groups of viruses that exhibit close morphological similarities but generally lack detectable genetic relationships is not unique to the herpesviruses, and may be explained as the result either of convergence

from distinct evolutionary sources or as divergence from an ancestor so ancient that sequence similarities have been obliterated. The latter hypothesis is currently favored, but the existence of a common ancestor of all herpesviruses and any contingent dates for divergence of the groups must be viewed cautiously. More speculatively, apparent similarities in aspects of DNA packaging (Booy *et al.*, 1991) and capsid maturation (Newcomb *et al.*, 1996) could be interpreted as supporting an even earlier common evolutionary origin between herpesviruses and certain double-stranded DNA bacteriophages, including T4.

Phylogenetic analyses strongly support the view that herpesviruses have largely co-evolved with their hosts, often co-speciating with them. As would be expected of evolutionary phenomena, a number of problematic observations and exceptions have emerged as data have multiplied, especially in relation to early divergences. From comparisons between the phylogenies of the viruses and their hosts, McGeoch and Cook (1994) proposed an evolutionary timescale for the *Alphaherpesvirinae* in which the *Simplex-* and *Varicellovirus* genera diverged about 73 million years ago, roughly coincident with the period of the mammalian radiation. Even at this stage, potential exceptions to the co-evolution model were apparent. For example, the taxonomical position of avian herpesviruses among the *Alphaherpesvirinae* did not fit well, and prompted the suggestion of ancient interspecies transfers between mammals and birds. In this scheme, a similar argument may be necessary to explain the position of reptilian (turtle) herpesviruses in the same subfamily (Quackenbush *et al.*, 1998; Yu *et al.*, 2001; Coberley *et al.*, 2002), especially given their distance from amphibian herpesviruses (Davison *et al.*, 1999 and unpublished data). Assuming the constancy of the molecular clock derived for the *Alphaherpesvirinae*, McGeoch *et al.* (1995) tentatively dated the divergence of the *Alpha-*, *Beta-* and *Gammaherpesvirinae* at 180–220 million years ago. Given the contrasting lack of relationships between the groups and substantial relationships within them (see below), this date did not fit well qualitatively with a model in which the fish and mammalian herpesvirus groups co-speciated when teleosts separated from other vertebrates. In a recent analysis utilizing improved algorithms and the latest estimates for host divergence dates, McGeoch and Gatherer (2005) pushed back the common ancestor of the *Alpha-*, *Beta-* and *Gammaherpesvirinae* to about 400 million years ago, which permitted a greater degree of support for co-evolution of the *Alphaherpesvirinae*, including avian and reptilian members. In this scheme, a much earlier, non-co-speciative divergence may be indicated for the mammalian and fish herpesvirus groups (along with one of similar or greater antiquity for the bivalve herpesvirus group). However, this would lack the advantage of explaining the segregation of the viral groups to distinct parts of the animal kingdom and necessitate additional arguments involving viral extinction.

The mammalian herpesvirus group

In contrast to the lack of extensive relationships between the three groups, members of the mammalian herpesvirus group are clearly related to each other (Davison, 2002), as are those in the fish herpesvirus group (Bernard and Mercier, 1993; Davison, 1998; Davison *et al.*, 1999; Waltzek *et al.*, 2005, and unpublished data). Figure 2.2 shows the gene layout in representatives of two genera for each of the three subfamilies in the mammalian herpesvirus group. The subfamilies share 43 genes, termed "core genes," which were presumably inherited from a common ancestor (McGeoch and Davison, 1999). This number assumes a small degree of approximation, since amino acid sequence conservation among the set varies from substantial to marginal. The core genes are shaded grey in Fig. 2.2, and are largely confined to the central regions of the genomes, as is especially apparent with HCMV. Accumulation of more recently evolved genes near the termini is a feature of linear, double-stranded DNA genomes from other virus families, such as the *Poxviridae* (Upton *et al.*, 2003; McLysaght *et al.*, 2003; Gubser *et al.*, 2004) and the *Adenoviridae* (Davison *et al.*, 2003c), and also of eukaryotic chromosomes (Kellis *et al.*, 2003). The core genes are ordered similarly in the same subfamily, except for certain members of the *Alphaherpesvirinae* in which different arrangements are apparent: PRV in the *Varicellovirus* genus (Ben-Porat *et al.*, 1983; Davison and Wilkie 1983; Dezelee *et al.*, 1996; Bras *et al.*, 1999) and ILTV in the *Iltovirus* genus (Ziemann *et al.*, 1998). However, as shown in Fig. 2.2, the core genes are arranged differently in the different subfamilies, in the form of blocks, some of which are inverted (Davison and Taylor, 1987; Gompels *et al.*, 1995; Hannenhalli *et al.*, 1995). As indicated in Table 2.2, core genes are involved in vital aspects of herpesvirus growth, and many are involved directly or indirectly in DNA replication, in packaging of replicated DNA into capsids, and in capsid formation and structure. Most of the core genes are essential for growth of virus in cell culture (Ward and Roizman, 1994; Yu *et al.*, 2003; Dunn *et al.*, 2003).

Most core genes are present in all three subfamilies of the mammalian herpesvirus group, but three (encod-

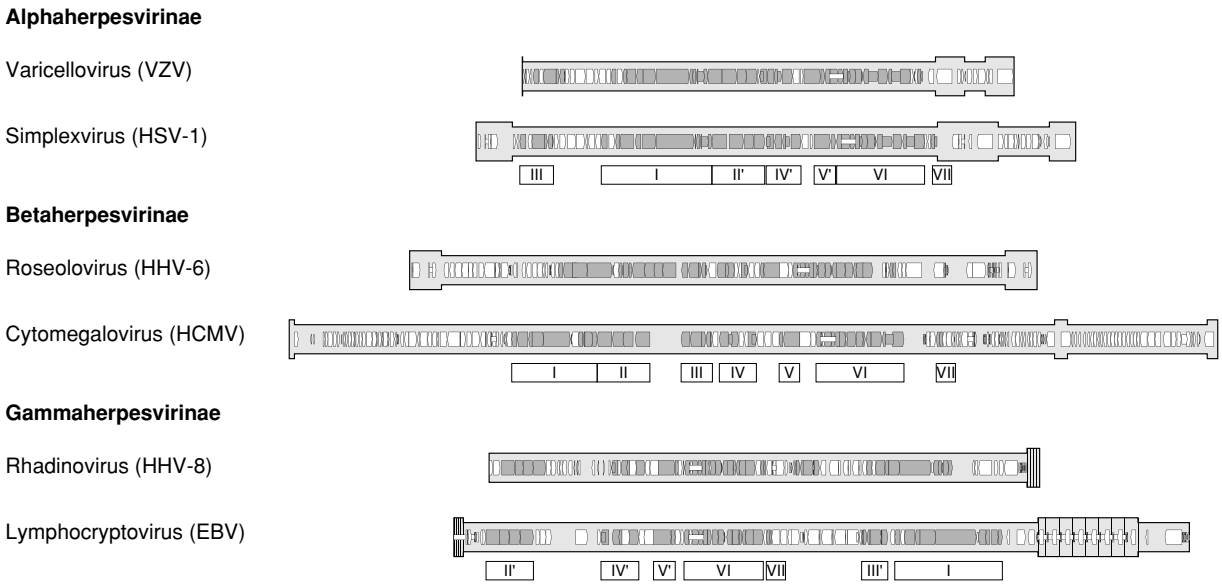

Fig. 2.2. Layout of genes in mammalian herpesvirus genomes. Repeat regions are shown in thicker format than unique regions. Protein-coding regions are shown as arrows shaded grey (core genes) or white (non-core genes), and introns as narrow white bars. Blocks of core genes that are rearranged between the subfamilies are indicated by rectangles I–VII for HSV-1, HCMV and EBV, with inverted blocks marked with a prime (Chee *et al.*, 1990). Block II also contains a local inversion and transposition of one gene (encoding DNA polymerase) that is not indicated. Genome coordinates and gene locations were obtained from accessions X04370 (VZV), X14112 (HSV-1), X83413 (HHV-6) as modified by Megaw *et al.* (1998), AY446894 (HCMV), U93872 (HHV-8) as extended at the right end of the unique region by Glenn *et al.* (1999), and AJ507799 (EBV). Variable numbers of terminal repeats are present in HHV-8 and EBV, but are shown at one end or the other other according to the accessions.

ing thymidine kinase, the small subunit of ribonucleotide reductase and the helicase that binds to the origin of DNA synthesis) have been lost from individual lineages. Thus, the origin-binding helicase gene has been retained in the *Roseolovirus* genus, but lost from other genera in the *Betaherpesvirinae*. This is mirrored in the presence of an origin of lytic DNA replication with similar structure in lineages that have retained this gene (Dewhurst *et al.*, 1993; Inoue *et al.*, 1994).

In addition to protein-coding regions, certain *cis*-acting sequences are conserved. These include the origin of lytic DNA replication, which is located similarly in each subfamily in comparison with adjacent genes, allowing for rearrangement of gene blocks. Certain members of the *Alpha-* and *Gammaherpesvirinae* contain additional lytic origins. Also, short elements near the genome termini that are involved in cleavage and packaging of unit-length genomes are conserved in all subfamilies (Broll *et al.*, 1999).

As well as the part played by the gradual processes of nucleotide substitution, insertion or deletion in generating diversity, acquisition of genes from the cell or from other viruses has played an important role throughout the evolution of the herpesviruses. There are examples of captured genes in all herpesvirus lineages. Among the mammalian herpesvirus group, the *Gammaherpesvirinae* exhibit a particularly impressive number of such genes, ranging from one encoding a product related to an enzyme involved in *de novo* purine biosynthesis (phosphoribosylformylglycineamide amidotransferase; FGARAT; Ensser *et al.*, 1997), which is present in all *Gammaherpesvirinae*, through a cyclin D gene (Nicholas *et al.*, 1992), which features in a subset of the *Rhadinovirus* genus, interferon regulatory factor genes (vIRFs), which are found only in HHV-8 and RRV (Russo *et al.*, 1996; Searles *et al.*, 1999), to a relatively recently captured core 2 β-1,6-*N*-acetylglucosaminyltransferase-mucin gene in BHV-4 (Markine-Goriaynoff *et al.*, 2003).

Duplication of genes, captured or otherwise, followed by divergence, is also apparent in all herpesvirus lineages. For example, up to three copies of the FGARAT gene are present in *Gammaherpesvirinae* (Virgin *et al.*, 1997), and HHV-8 and RRV contain four and eight vIRF genes, respectively (Searles *et al.*, 1999; Jenner *et al.*, 2001; Cunningham *et al.*, 2003). Examples of duplicated genes among other members of the mammalian herpesvirus group include

Table 2.2. Core genes in human herpesviruses, grouped according to functional class. HSV-2 and HHV-7 are not included, since their nomenclatures are the same as those for HSV-1 and HHV-6, respectively. HSV-1 and HCMV genes that are essential for growth in cell culture are marked by asterisks

HSV-1	VZV	HCMV	HHV-6	EBV	HHV-8	Function
DNA replication machinery						
UL30*	28	UL54*	U38	BALF5	9	Catalytic subunit of DNA polymerase complex
UL42*	16	UL44*	U27	BMRF1	59	Processivity subunit of DNA polymerase complex
UL9*	51	–	U73	–	–	Origin-binding protein; helicase
UL5*	55	UL105*	U77	BBLF4	44	Component of DNA helicase-primase complex; helicase
UL8*	52	UL102*	U74	BBLF2/BBLF3	40/41	Component of DNA helicase-primase complex
UL52*	6	UL70*	U43	BSLF1	56	Component of DNA helicase-primase complex; primase
UL29*	29	UL57*	U41	BALF2	6	Single-stranded DNA-binding protein
Peripheral enzymes						
UL23	36	–	–	BXLF1	21	Thymidine kinase
UL39	19	UL45[a]	U28[a]	BORF2	61	Ribonucleotide reductase; large subunit
UL40	18	–	–	BaRF1	60	Ribonucleotide reductase; small subunit
UL50	8	UL72[a]	U45[a]	BLLF3	54	Deoxyuridine triphosphatase
UL2	59	UL114	U81	BKRF3	46	Uracil-DNA glycosylase
Processing and packaging of DNA						
UL12	48	UL98*	U70	BGLF5	37	Deoxyribonuclease; role in DNA maturation and recombination
UL15*	42/45	UL89*	U66	BGRF1/BDRF1	29	Putative ATPase subunit of terminase; capsid-associated
UL28*	30	UL56*	U40	BALF3	7	Putative subunit of terminase; *pac* site-specific binding; capsid-associated
UL6*	54	UL104*	U76	BBRF1	43	Portal protein; forms dodecameric ring at capsid vertex; complexed with terminase
UL25*	34	UL77*	U50	BVRF1	19	Possibly caps the portal after DNA packaging is complete; tegument protein
UL32*	26	UL52*	U36	BFLF1	68	Involved in proper capsid localization in the nucleus
UL33*	25	UL51*	U35	BFRF1A	67A	Interacts with terminase
UL17*	43	UL93*	U64	BGLF1	32	Involved in proper capsid localization in the nucleus; tegument protein
Egress of capsids from nucleus						
UL31*	27	UL53*	U37	BFLF2	69	Nuclear matrix protein; component of capsid docking complex on nuclear lamina
UL34*	24	UL50*	U34	BFRF1	67	Inner nuclear membrane protein; component of capsid docking complex on nuclear lamina
Capsid assembly and structure						
UL19*	40	UL86*	U57	BcLF1	25	Major capsid protein; component of hexons and pentons
UL18*	41	UL85*	U56	BDLF1	26	Component of intercapsomeric triplex between hexons and pentons
UL38*	20	UL46*	U29	BORF1	62	Component of intercapsomeric triplex between hexons and pentons
UL35	23	UL48A*	U32	BFRF3	65	Small capsid protein located on tips of hexons; interacts with dynein and microtubules
UL26*	33	UL80*	U53	BVRF2	17	Maturational protease; generates mature forms of scaffolding proteins
UL26.5	33.5	UL80.5	U53.5	BdRF1	17.5	Scaffolding protein removed from capsid during DNA packaging
Tegument						
UL7	53	UL103	U75	BBRF2	42	Associated with intracellular capsids
UL11	49	UL99*	U71	BBLF1	38	Role in virion egress and secondary envelopment in the cytoplasm; myristylated and palmitylated protein; interacts with UL16 protein
UL14	46	UL95*	U67	BGLF3	34	Interacts with UL11 protein; regulates UL13 protein kinase
UL16	44	UL94*	U65	BGLF2	33	Interacts with UL11 protein; regulates UL13 protein kinase
UL21[b]	38	UL88	U59	BTRF1	23	
UL36*	22	UL48*	U31	BPLF1	64	Huge virion protein; interacts with UL37 protein; influences release of DNA from capsids during entry

Table 2.2. (*cont.*)

HSV-1	VZV	HCMV	HHV-6	EBV	HHV-8	Function
UL37*	21	UL47	U30	BOLF1	63	Interacts with UL36 protein
UL51	7	UL71*	U44	BSRF1	55	
Surface and membrane						
UL27*	31	UL55*	U39	BALF4	8	gB
UL1*	60	UL115*	U82	BKRF2	47	gL; complexed with gH
UL22*	37	UL75*	U48	BXLF2	22	gH; complexed with gL
UL10	50	UL100*	U72	BBRF3	39	gM; complexed with gN
UL49A[c]	9A	UL73*	U46	BLRF1	53	gN; complexed with gM; not glycosylated in some herpesviruses
Control and modulation						
UL13	47	UL97	U69	BGLF4	36	Serine–threonine protein kinase; tegument protein
UL54*	4	UL69	U42	BSLF1/BMLF1	57	Multifunctional regulator of gene expression
Unknown						
UL24	35	UL76*	U49	BXRF1	20	Nuclear protein

[a] Probably not an active enzyme, as catalytic residues are absent.
[b] This assignment is tentative and is excluded from the total of 43 core genes given in the text. It depends on positional, rather than sequence, conservation, and is compromised by that fact that UL21 is not flanked in each subfamily by clear homologues, unlike other core genes assigned on a positional basis.
[c] Also referred to as UL49.5.

a family of glycoprotein genes in the *Alphaherpesvirinae* (McGeoch, 1990) and 12 families, each containing up to 14 genes, in HCMV as a representative of the *Betaherpesvirinae* (Dolan *et al.*, 2004). There are four gene families in the fish herpesvirus, CCV (Davison, 1992), and 12 in the bivalve herpesvirus, OsHV-1 (Davison *et al.*, 2005). Given that gene duplication has been widely employed in host evolution (Prince and Pickett, 2002), and the greater evolutionary rates of herpesviruses, it seems likely that this means for generating diversity has played a greater part in herpesvirus evolution than can be detected by primary sequence comparisons.

The *Alphaherpesvirinae* subfamily

The employment of gene capture and duplication among the *Beta-* or *Gammaherpesvirinae* to generate diversity has received extensive attention in the literature (for details, see Chapters 15 and 22). In contrast, the *Alphaherpesvirinae* have evolved less adventurously in terms of gene content since their divergence from a common ancestor, and it is clear that gene loss has occurred. This mode of survival is considered in the following paragraphs.

The *Alphaherpesvirinae* contain four genera, plus the reptilian herpesviruses. Several complete genomes have been sequenced for the *Simplexvirus*, *Varicellovirus* and *Mardivirus* genera (Table 2.1). Data for the *Iltovirus* genus are more sparse. Limited sequence data are available for reptilian herpesviruses. Figure 2.3 illustrates the genetic content of members of the *Varicello-*, *Simplex-* and *Mardivirus* genera. Three examples (VZV, EHV-1 and BHV-1) were chosen to represent the major lineages in the *Varicellovirus* genus (see Chapter 1), plus SVV as a close relative of VZV. Core genes are shown in grey, and other genes that have counterparts in all three genera are shown in white. All of these genes were presumably present in the common ancestor, which is estimated to have existed 135 million years ago (McGeoch and Gatherer, 2005), and they comprise all or nearly all of the genes in extant *Alphaherpesvirinae*. It seems that only a few genes have developed since that era, and that the most of these are located near the genome termini.

Figure 2.4 shows a scheme of relationships between the genes at the left end of the genome, based on sequence conversation and the observation that two genes (UL56 and ORF1) encode potential membrane proteins. Also included are data for PRV (whose closest sequenced relative is BHV-1), MDV-2 and ILTV. Since U_L inverts in HSV-1, and U_L in the prototype genome orientation turned out to be inverted in comparison with the *Varicellovirus* genus (Hayward *et al.*, 1975; Wadsworth *et al.*, 1975), the genes at the left end of HSV-1 U_L are presented in the reverse order. In Fig. 2.4,

Fig. 2.3. Layout of genes in genomes of the *Alphaherpesvirinae*. Repeat regions are shown in thicker format than unique regions. Protein-coding regions are shown as arrows shaded grey (core genes), white (other genes shared by two or more genera) or black (other genus-specific genes that have presumably evolved more recently), and introns as narrow white bars. Genome coordinates and gene locations were obtained from accessions listed in the legend to Fig. 2.2, and from AF275348 (SVV), M86664 (EHV-1), AJ004801 (BHV-1) and AF243438 (MDV-1).

UL56 and UL55 thus precede UL54, which is a core gene. VZV has two extra genes (ORF1 and ORF2) sandwiched between UL55 and UL56, and the other viruses have between one and four genes in this region. For example, SVV lacks ORF2 and has a partial duplication of UL54 near the end of the genome. A parsimonious approach indicates that the ancestor preceding divergence of the *Iltovirus* genus had at least one of the genes at the left genome terminus (UL56; 180 million years ago; McGeoch and Gatherer, 2005), that the ancestor preceding divergence of the *Mardivirus* genus had at least three (UL56 (since lost), UL55 and ORF2; 135 million years ago), that the ancestor of the *Varicellovirus* and *Simplexvirus* genera had at least three (UL56, UL55 and ORF2; 120 million years ago) and that the ancestor of VZV and EHV-1 had all four genes (82 million years ago). Various of these genes have been lost during subsequent evolution of the mammalian viruses.

Gene loss is also apparent at the right genome terminus (Fig. 2.4), where, again, few genes are specific to one virus or a few closely related viruses. Even the more recently evolved genes may have substantial histories. Of the three HSV-1 genes in this category, two at the right end of U_S (US11 and US12) have counterparts in related viruses of monkeys (HVB and SA8; Ohsawa *et al.*, 2002; Tyler *et al.*, 2005). HSV-1 and HVB are considered to have co-speciated with their hosts about 23 million years ago (McGeoch *et al.*, 2000). The gene at the left genome terminus (RL1; repeated internally) has a counterpart at a similar location in a wallaby herpesvirus genome (Guliani *et al.*, 2002).

Outlook

Investigation of the genome structures, genetic contents and evolution of herpesviruses is a maturing field that undergirds the rest of herpesvirology. As with other complex analytical subjects, future advances will require incisive examination of both new data and the framework into which they are fitted. There is yet room for more surprises.

"It is a mistake to try to look too far ahead. The chain of destiny can only be grasped one link at a time." *Winston Churchill.*

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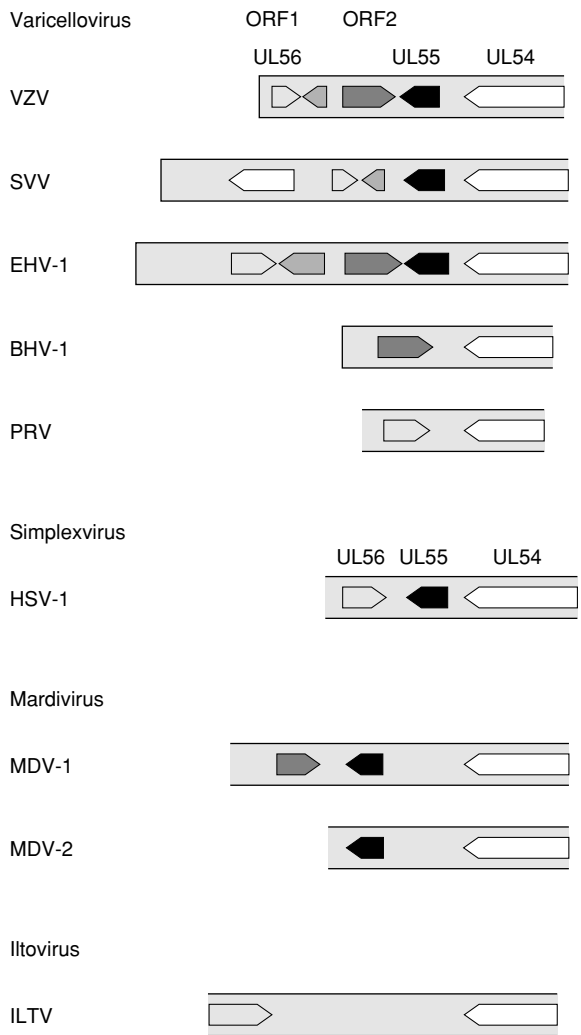

Fig. 2.4. Layout of genes at or near the left terminus in genomes of the *Alphaherpesvirinae*. The left terminus, where included, is shown by a vertical line. Homologous genes are shaded equivalently, and their nomenclature is indicated. An additional non-homologous gene is present in the *Mardi-* and *Iltovirus* genomes down stream from UL55, but is not shown. Gene locations were derived or deduced from accessions listed in the legend to Fig. 2.3, and from BK001744 (PRV), AB049735 (MDV-2) and U80762 (ILTV).

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3

Comparative virion structures of human herpesviruses

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Introduction

The herpesvirus family consists of a group of viruses distinguished by the large size of their linear double-stranded DNA genomes (~130–250 kbp) and a common architecture of infectious particles (Fig. 3.1) (Chiu and Rixon, 2002; Gibson, 1996; Steven and Spear, 1997). Indeed, before the birth of molecular biology and the availability of genomic sequencing, the common hallmark structural features shared by these viruses were the most important criteria for the classification of a herpesvirus (Roizman and Pellett, 2001). All herpesviruses identified to date, which include eight different types that are known to infect human, and more than 170 other viruses that are found in animals as well as in fish and amphibians (Roizman and Pellett, 2001), exhibit identical structural design as illustrated using human cytomegalovirus shown in Fig. 3.1. These viruses have a highly ordered icosahedral-shape nucleocapsid of about 125–130 nm in diameter, which encases the viral DNA genome. The nucleocapsid is surrounded by a partially ordered proteinaceous layer called the tegument, which in turn is enclosed within the envelope, a polymorphic lipid bilayer containing multiple copies of more than 10 different kinds of viral glycoproteins that are responsible for viral attachment and entry to host cells.

Based on their biological properties such as growth characteristics and tissue tropism, herpesviruses can be further divided into three subfamilies. Among the eight human herpesviruses, the alpha subfamily includes neurotropic viruses and contains the herpes simplex virus (HSV) 1 and 2, and Varicella zoster virus (VZV). The members of the gamma subfamily are lymphotropic viruses and include Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). The viruses of the beta subfamily appear to be able to establish infections in many different types of cells and tissues, and include human cytomegalovirus (HCMV), and human herpesvirus 6 and 7. This subfamily classification system is largely consistent with the extensive genomic information that is now available (McGeoch *et al.*, 2000). While studies have been attempted to investigate the structure and architecture of each of the eight human herpesviruses, virion and virus-related particles of herpes simplex virus 1 (HSV-1), the prototype of all herpesviruses, have been subjected to the most extensive structural studies (Booy *et al.*, 1991; Newcomb *et al.*, 1993, 2000; Schrag *et al.*, 1989; Trus *et al.*, 1996; Zhou *et al.*, 1999, 2000). During the last several years, significant progress has also been made in understanding the structure of cytomegaloviruses (Bhella *et al.*, 2000; Chen *et al.*, 1999; Trus *et al.*, 1999), the prototype of the beta herpesvirus family, and KSHV, a representative of the gamma herpesvirus family (Lo *et al.*, 2003; Nealon *et al.*, 2001; Trus *et al.*, 2001; Wu *et al.*, 2000). Using HSV-1, HCMV, and KSHV as examples for each of the subfamilies, this chapter focuses primarily on the structures of these three viruses, and discusses the recent progress on understanding the structures of human herpesviruses.

Different virus-related particles found in infected cells

Summary of virion assembly pathway

Each of the herpesviruses encodes a specific set of proteins that form the different compartments of the virion (e.g. capsid, Table 3.1). Although many of the primary amino acid sequences of these proteins are not highly conserved among different viruses, the assembly pathway of the virus particles is highly similar (Fig. 3.2) (Gibson, 1996; Roizman and Knipe, 2001; Yu *et al.*, 2003). The nucleocapsid

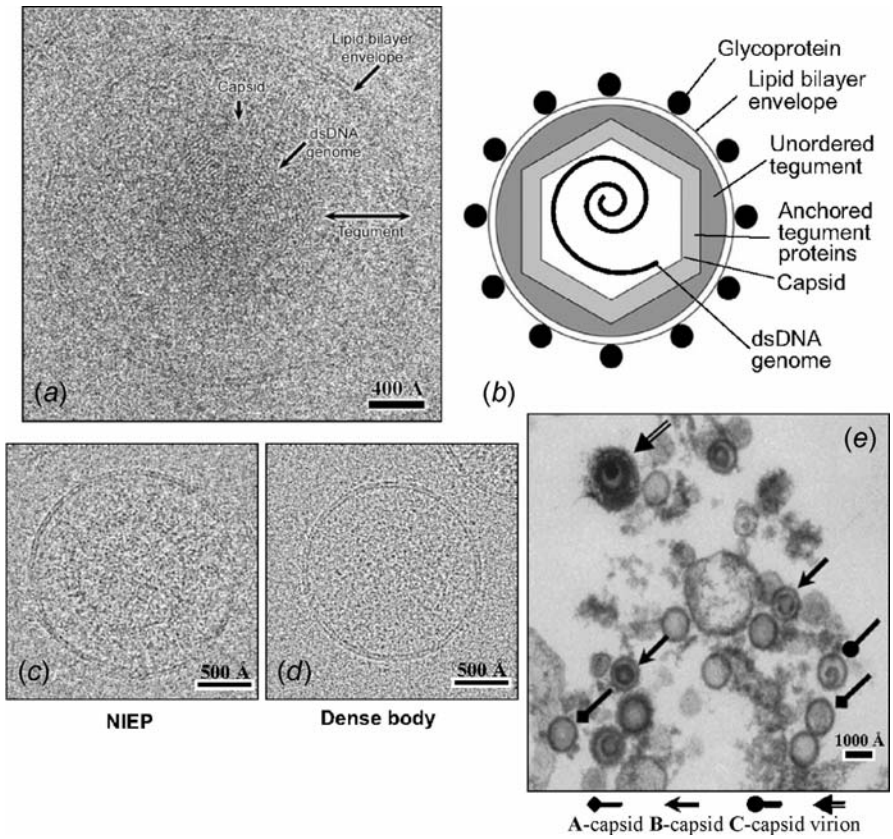

Fig. 3.1. Herpesvirus architecture. (*a*) Electron cryomicrograph of a human cytomegalovirus virion showing the different compartments of a herpes virion. (*b*) Schematic diagram illustrating the multilayer organization of human herpesviruses. Also shown are the electron cryomicrograph of a non-infectious enveloped particle (NIEP) (*c*) and a dense body (*d*) isolated from HCMV virion preparations. (*e*) Virions and different kinds of capsids observed in the thin sections of human foreskin fibroblasts infected with HCMV by negative stain electron microscopy.

is formed in the nucleus and follows a pathway that bears a marked resemblance to those of DNA bacteriophages (Casjens and Hendrix, 1988). First, a procapsid is assembled with the formation of the capsid shell and the internal scaffolding structure. Second, the procapsid is converted into mature nucleocapsid, during which time, the morphogenic internal scaffolding protein is released and replaced by the viral DNA genome, concomitant with a major conformation change of the capsid shell (Newcomb *et al.*, 1999; Yu *et al.*, 2005). Subsequent events, however, differ from the phage assembly pathway (Fig. 3.2). The mature nucleocapsid exits the nucleus and acquires its tegument and envelope, through repeated fusion with and detachment from nuclear membranes and other cellular membranous structures. Eventually, the mature infectious virion particles are released into the extracellular space via cellular secretory pathways. During this assembly process, different virus-related particles and structures, including the mature nucleocapsids and virions as well as the intermediate and aberrant products, can be found in the infected cells and the extracellular media (Figs. 3.1(*c*)–(*e*) and 3.2).

Different virus-like particles secreted from infected cells

Since the discovery of the herpesviruses, it has been long recognized that, in addition to producing infectious virus particles, the infected host cells also generate noninfectious particles such as noninfectious enveloped particles (NIEP, Fig. 3.1(*c*)) and dense bodies (DB) (Figs. 3.1(*d*) and 3.2) (Gibson, 1996; Steven and Spear, 1997). Both NIEP and DB are commonly found in the culture media of cells that are lytically infected with HSV-1 and HCMV. The ratio

Table 3.1. Major virion proteins present in HSV-1, HCMV and KSHV

		HSV-1			HCMV			KSHV			
Location	Common name	Protein name	ORF	Size (aa)	Protein name	ORF	Size (aa)	Protein name	ORF	Size (aa)	
inside the capsid	protease	protease	UL26	635	NP1c	UL80a	708	Pr	ORF17	553	~100
	Scaffolding	VP22a	UL26.5	329	AP	UL80.5	373	AP	ORF17.5	283	~1200 in B-capsid, 0 in A- & C-capsids
	MCP	VP5	UL19	1374	MCP	UL86	1370	MCP	ORF25	1376	960; penton & hexon subunit
on the Capsid shell	TRI-2	VP23	UL18	318	mCP	UL85	306	TRI-2	ORF26	305	640, dimer in triplex
	TRI-1	VP19c	UL38	465	mCBP	UL46	290	TRI-1	ORF62	331	320, monomer in triplex
	SCP	VP26	UL35	112	SCP	UL48.5	75	SCP	ORF65	170	900; hexon tip

Abbreviations: MCP, major capsid protein; TRI-2, triplex dimer protein; TRI-1, triplex monomer protein; SCP, smallest capsid protein; Pr, protease; AP, assembly protein.

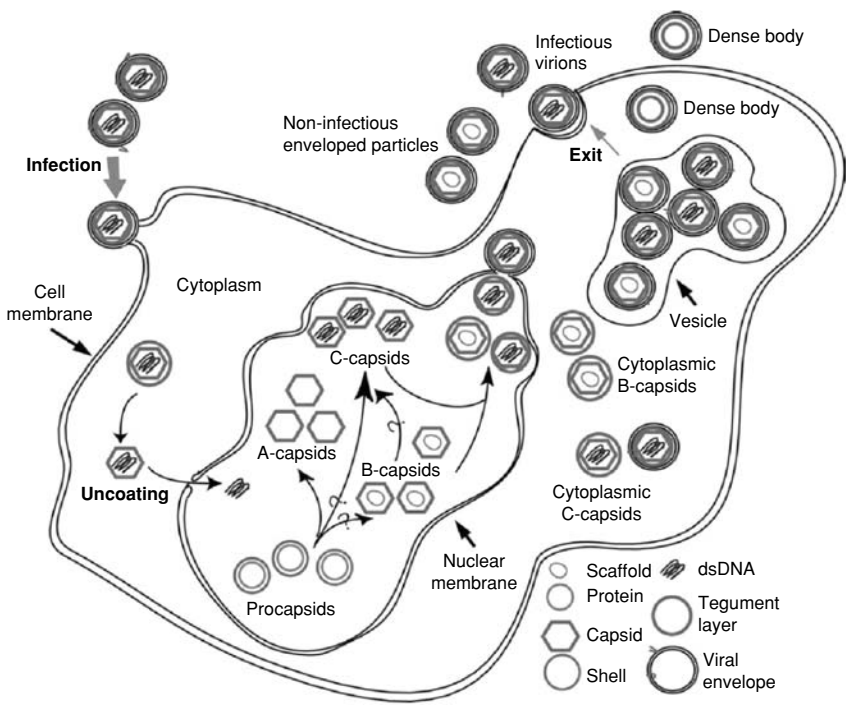

Fig. 3.2. Different virus-like particles and structures during lytic cycle of herpesvirus replication. The infectious virion initializes infection by either endocytosis or fusion with the cell membrane, which releases the nucleocapsid and some tegument proteins into the cytoplasm. The nucleocapsid is uncoated and transported across the cytoplasm (Sodeik *et al.*, 1997), allowing injection of the viral DNA through nuclear pores into the nucleus, where replication and capsid assembly take place. Procapsids mature into the C-capsid by encapsidating the viral dsDNA. Failure of DNA encapsidation results in the abortive A-capsid. Both B-capsid and C-capsid can acquire a layer of tegument proteins at the nuclear membrane of the host cell to become cytoplasmic capsids, and are enveloped and released by exocytosis to become non-infectious (NIEP) and infectious particles, respectively. Dense bodies, which contain a large amount of tegument proteins but no capsids or viral DNA, can also be found in the extracellular media.

of these particles to mature infectious virion particles can sometimes reach 20:1, suggesting that they are produced in great excess (Gibson, 1996; Steven and Spear, 1997). The exact function of these non-infectious particles in viral infection and replication is currently unknown, although they have been proposed to act as decoys that saturate and overwhelm the immune surveillance thereby facilitating the survival of the infectious virions in the hosts (Gibson, 1996; Steven and Spear, 1997).

Structurally, both the NIEP and DB are significantly different from the infectious virion (*cf.* Fig. 3.1(*a*), (*c*), (*d*)). They can be easily distinguished using electron cryomicroscopy (cryoEM) and separated from the mature infectious virions using ultracentrifugation approaches. As described above, all infectious herpesvirus virions share four common structural features (Fig. 3.1(*b*)). First, all herpesviruses contain a large double-stranded DNA (dsDNA) genome. The genomic DNA represents a dense core of ~90 nm in diameter, which can be stained with uranyl acetate and visualized using electron microscopy (Gibson, 1996; Steven and Spear, 1997) and appears as "fingerprint" patterns when examined by electron cryomicroscopy (Fig. 3.1(*a*)) (Booy *et al.*, 1991; Zhou *et al.*, 1999). Second, a capsid of icosahedral shape, which primarily consists of many copies of four different viral proteins, encases the genomic DNA. Third, a protein layer structure, named as the tegument first by Roizman and Furlong (Roizman and Furlong, 1974), surrounds the capsid and occupies the space between the capsid and the envelope. The tegument structure contains many virus-encoded factors that are important for initiating viral gene transcription and expression as well as modulating host metabolism and shutting down host antiviral defense mechanism (for a brief review, see Roizman and Sears, 1996). Finally, a lipid-bilayer envelope constitutes the outermost perimeters of the particles, and contains all the surface virion glycoproteins that are responsible for viral infectivity and entry (Fig. 3.1).

Unlike infectious virion particles, a NIEP does not contain a genomic DNA core and its capsid core appears to be B-capsid-like under electron microscopy (Figs. 3.1(*c*) and 3.2). In contrast, a dense body does not contain a capsid and appears as a cluster of tegument proteins encased by the lipid-bilayer membranous envelope (Fig. 3.1(*d*)). The presence of NIEP and DB indicates that neither packaging of viral genome nor capsid formation is required for viral envelopment.

Different capsid-like structures inside the infected cells

The capsid assembly is a continuous sequential process, leading to the synthesis of the highly ordered capsid structures. In cells that are lytically infected with herpesviruses, several kinds of virus capsid-like structures have been identified as representing stable endpoints or long-lived states (Figs. 3.1 and 3.2). Gibson and Roizman first introduced the terms A-, B-, and C-capsids to describe these intracellular capsid-like structures in HSV-1 infected cells (Gibson and Roizman, 1972). Similar capsid structures have been observed in cells infected with HCMV (Gibson, 1996; Irmiere and Gibson, 1985). Recent work has revealed A-, B- and C-capsids of comparable chemical composition and structural features in the nuclei of gammaherpesvirus infected cells and this suggests that the gammaherpesvirus capsid assembly probably also proceeds in a similar manner (O'Connor *et al.*, 2003; Yu *et al.*, 2003). These capsids all have a distinctive polyhedral shape when examined under electron microscope. Another capsid type, termed procapsid, can be obtained from in vitro assembly experiments using recombinant capsid proteins or from cells infected by a HSV-1 mutant containing a temperature sensitive mutation at the gene encoding the viral protease (Rixon and McNab, 1999; Trus *et al.*, 1996). The procapsid has a distinctive spherical shape and is only transiently stable. They undergo spontaneous structural rearrangement to become the stable angular or polyhedral form similar to the other types of capsids (Heymann *et al.*, 2003; Yu *et al.*, 2005; Zhou *et al.*, 1998b). A-capsids represent empty capsid shells that contain neither viral DNA nor any other discernible internal structure. They are thought to arise from abortive, dead-end products derived from either the inappropriate loss of viral DNA from a C-capsid or the premature release of scaffolding protein from a B-capsid without concurrent DNA packaging (Gibson, 1996). B-capsids are capsid shells containing an inner array of scaffolding protein. C-capsids are mature capsid shells that are packaged with viral DNA and do not contain the scaffolding proteins. B-capsids are believed to be derived from the procapsids upon proteolytic cleavage of the scaffolding protein, and their fate in viral maturation is controversial. Early pulse-chase experiments have suggested that B-capsids can mature to C-capsids, which in turn serve as the infectious virus precursors (Perdue *et al.*, 1976), and recent studies suggest that they might also be a dead-end product in capsid assembly similar to A-capsids (Trus *et al.*, 1996; Yu *et al.*, 2004). It remains unclear whether the spherical procapsids first adapt to the stable angular form before or after the cleavage of its scaffolding protein. The C-capsid buds through the nuclear membrane using an envelopment and de-envelopment process and acquires an additional layer of proteins that forms the tegument in the cytoplasm (for review, see Mettenleiter, 2002). Enveloped virions are then released by exocytosis (Fig. 3.2).

Assembly of viral capsid

A-, B- and C-capsids represent the stable intermediates or the end products of the herpesvirus capsid assembly process (Figs. 3.1(*e*) and 3.2). In HSV-1, capsid assembly begins with the formation of the spherical procapsid through the association of the carboxyl terminus of the scaffolding protein with the amino terminus of the viral major capsid protein (MCP), similar to bacteriophage proheads (Conway *et al.*, 1995; Jiang *et al.*, 2003). Previous experiments have shown that the procapsid can be assembled in vitro from the capsid and scaffolding proteins, in the absence of the viral capsid maturation protease (Newcomb *et al.*, 1999) or from cells infected with viruses containing a temperature-sensitive protease mutant (Heymann *et al.*, 2003). These procapsids can spontaneously rearrange into a large-cored, angular particle resembling the B-capsid, but these large-cored particles do not encapsidate DNA or become mature virions. Past studies have also shown that cells infected with a HSV-1 mutant containing a temperature-sensitive mutation in the protease gene produced capsids that assemble at the non-permissive temperature, similar to the in vitro-assembled procapsids (Rixon and McNab, 1999). The capsids matured when protease activity was restored (Rixon and McNab, 1999), demonstrating that the procapsid is the precursor to the angular capsid (Fig. 3.2). The proteolytic cleavage of the intra-capsid scaffolding proteins at their C-termini by the viral protease (Hong *et al.*, 1996; Liu and Roizman, 1991, 1992; Preston *et al.*, 1992; Welch *et al.*, 1991) interrupts the interactions between the scaffolding proteins and the major capsid proteins (Zhou *et al.*, 1998b). The interactions between the scaffolding protein, the major capsid protein, and viral protease are important targets for antiviral drug design in treating and controlling herpesvirus infections (Flynn *et al.*, 1997; Qiu *et al.*, 1996; Shieh *et al.*, 1996; Tong *et al.*, 1996, 1998). Proteolytic cleavage of the scaffolding protein is followed by the recruitment of the smallest capsid protein, VP26, through an ATP-dependent process (Chi and Wilson, 2000), leading to the formation of the intermediate or B-capsids. The mature procapsids are believed to arise spontaneously by packaging the viral genome DNA, a process that is currently not completely understood (Yu *et al.*, 2005).

Compositions and three-dimensional structural comparisons of alpha, beta and gammaherpesvirus capsids

A-, B-, and C-capsids (Yu *et al.*, 2005) can be isolated from the nucleus of the host cells lytically infected by herpesviruses and they have been subjected to three-dimensional structure studies for HSV-1 (Zhou *et al.*, 1998a, 1994), HCMV (Butcher *et al.*, 1998; Chen *et al.*, 1999; Trus *et al.*, 1999), and KSHV (Nealon *et al.*, 2001; Trus *et al.*, 2001; Wu *et al.*, 2000; Yu *et al.*, 2003). While these three types of capsids have different composition (e.g., viral DNA and internal scaffolding protein), they all have a common shell structure that consists of 150 hexameric (hexon) and 12 pentametric (penton) capsomers, which are connected in groups of three by the triplexes, asymmetric structures that lie on the capsid floor (Fig. 3.3). During the last few years, considerable progress of the three-dimensional structure of the capsids and the assembly of the capsomers and triplexes has been made on the studies.

The capsid, approximately 1250–1300 Å in diameter, is a $T = 16$ icosahedron with 12 pentons forming the vertices, 150 hexons forming the faces and edges, and 320 triplexes interconnecting the pentons and hexons (Rixon, 1993; Steven and Spear, 1997). One of the 20 triangular faces of the icosahedral capsid is indicated by the dotted triangle in Fig. 3.3(*a*) with three fivefold ('5'), a twofold ('2') and threefold (through triplex Tf) symmetry axes labeled. The six fivefold axes pass through the vertices, the ten threefold (3f) axes pass through the centers of the faces, and the 15 twofold (2f) axes pass through the middle of the edges. The structural components in one asymmetric unit are labeled, including 1/5 of a penton ('5'), one P (peri-pentonal) hexon, one C (center) hexons a half E (edge) hexon (Steven *et al.*, 1986), and one each of Ta, Tb, Tc, Td and Te triplex and 1/3 of Tf triplex (Fig. 3.3(*a*)) (Zhou *et al.*, 1994).

HSV-1 is the easiest to grow among all human herpesviruses and has been subjected to the most thorough structural analyses, and its capsid has been reconstructed to 8.5 Å resolution (Fig. 3.3(*a*)) (adapted from Zhou *et al.*, 2000 with permission from the publisher). The capsid shell has a total mass of about 200 MDa. The structural features of the capsid are built from four of the six capsid proteins: 960 copies of the major capsid protein (MCP), VP5; 320 copies of triplex monomer protein (TRI-1), VP19c; 640 copies of triplex dimer protein (TRI-2), VP23; and 900 copies of the smallest capsid protein (SCP), VP26. At this high resolution, details of secondary structure can be resolved that are not visible at lower resolution. Alpha-helices, for example, appear as extended, cylindrical rods of 5–7 Å diameter. The VP5 major capsid protein of HSV-1 was found to contain 24 helices. These assignments of helices to densities were corroborated by docking the cryoEM structure with X-ray crystallographic data which were subsequently obtained for the upper domain of VP5 (Fig. 3.3(*c*)) (Baker *et al.*, 2003; Bowman *et al.*, 2003). A group of seven helices is clustered near the area of the protein that forms the narrowest part of the

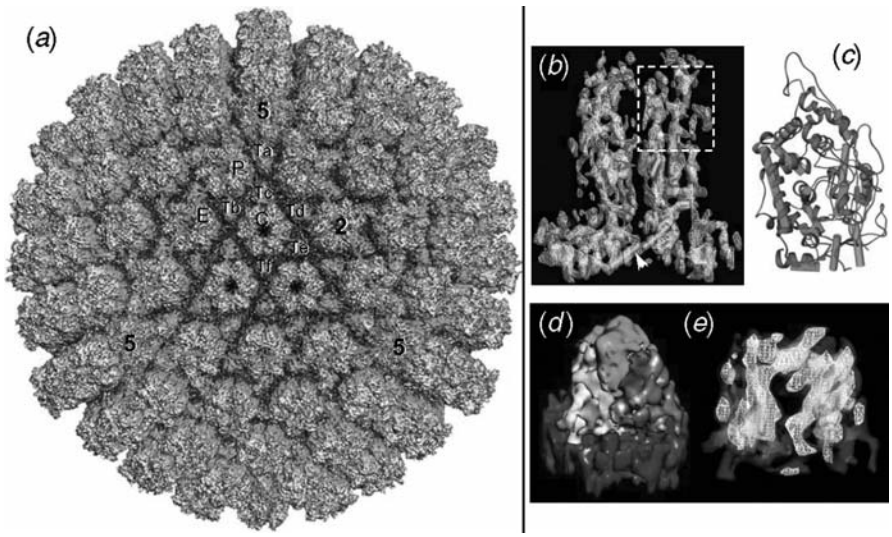

Fig. 3.3. HSV-1 capsid at 8 Å resolution (Zhou *et al.*, 2000) and atomic model of upper domain of the major capsid protein (MCP), VP5 (Bowman *et al.*, 2003). (*a*) Radially color-coded surface representation of the HSV-1 B capsid structure at 8.5 Å. One of the 20 triangular faces is denoted by dashed triangle. The penton and three types of hexons are indicated by '5', P, E and C. Also labeled are the six quasi-equivalent triplexes, Ta, Tb, Tc, Td, Te, Tf. (*b*) Two hexon subunits were shown in wire frame representation with α helices identified in one of the VP5 subunit illustrated by orange cylinders (5 Å in diameter). The red arrowhead points to the 7 helix bundle in the middle domain and the white arrow identifies the long helix in the floor domain that connects adjacent subunits.
(*c*) Ribbon representation of the atomic structure of the HSV-1 MCP upper domain determined by X-ray crystallography (Bowman *et al.*, 2003). The helices identified in the hexon VP5 subunit in the 8.5 Å HSV1 capsid map (Zhou *et al.*, 2000) are shown as cylinders: those in green match with helices present in the X-ray structure and those in yellow are absent in the X-ray model, suggesting possible structural differences of MCP packed in the crystal and inside the virion. (*d*) One single triplex is shown as shaded surface representation with individual subunits in different colors: VP19c in green and the two quasi-equivalent VP23 subunits in light and dark grey, all situated on the capsid shell domains of VP5 (blue). (*e*) α-helices identified in the two quasi-equivalent VP23 molecules (in red and yellow cylinders of 5 Å diameter, respectively). Adapted with permissions from publishers. (See color plate section.)

axial channel of the pentons and hexons (indicated by the red arrowhead in Fig. 3.3(*b*)). Shifts in these helices might be responsible for the constriction that closes off the channel to prevent release of packaged DNA. The floor domain of VP5 also contains several helices, including an unusually long one that interacts with the scaffolding core and may also interact with adjacent subunits to stabilize the capsid (arrow in Fig. 3.3(*b*)). Structural studies of in vitro assembled capsids that are representatives of capsid maturation stages suggest that substantial structural rearrangement at this region is directly related to the reinforcement of penton and hexons during morphogenesis (Heymann *et al.*, 2003).

The higher resolution of this reconstruction also revealed the quaternary structure of the triplexes, which are composed of two molecules of VP23 and one molecule of VP19c (Fig. 3.3(*d*), (*e*)). The lower portion of the triplex, which interacts with the floor of the pentons and hexons, are threefold symmetric with all three subunits roughly equivalent. This arrangement alters through the middle of the triplex such that the upper portion is composed mostly of VP23 in a dimeric configuration. It appears that all three subunits of the triplex are required for the correct tertiary structure to form because VP23 in isolation exists only as a molten globule with no distinct tertiary structure (Kirkitadze *et al.*, 1998).

The capsids of other human herpesviruses have also been studied by electron cryomicroscopy, including HCMV and simian cytomegalovirus (SCMV), and KSHV, members of the beta and gammaherpesviruses, respectively (Fig. 3.4) (Bhella *et al.*, 2000; Chen *et al.*, 1999; Trus *et al.*, 1999, 2001; Wu *et al.*, 2000). The HCMV capsid structure is very similar to HSV-1 in overall organization, with four homologous structural proteins at the same stoichiometries (Fig. 3.4(*a*) and (*b*)). The main difference is that the HCMV capsid had a larger diameter (650 Å) than HSV-1 (620 Å), resulting in a volume ratio of 1.17 (Bhella *et al.*, 2000; Chen *et al.*, 1999; Trus *et al.*, 1999). The increased size of the HCMV

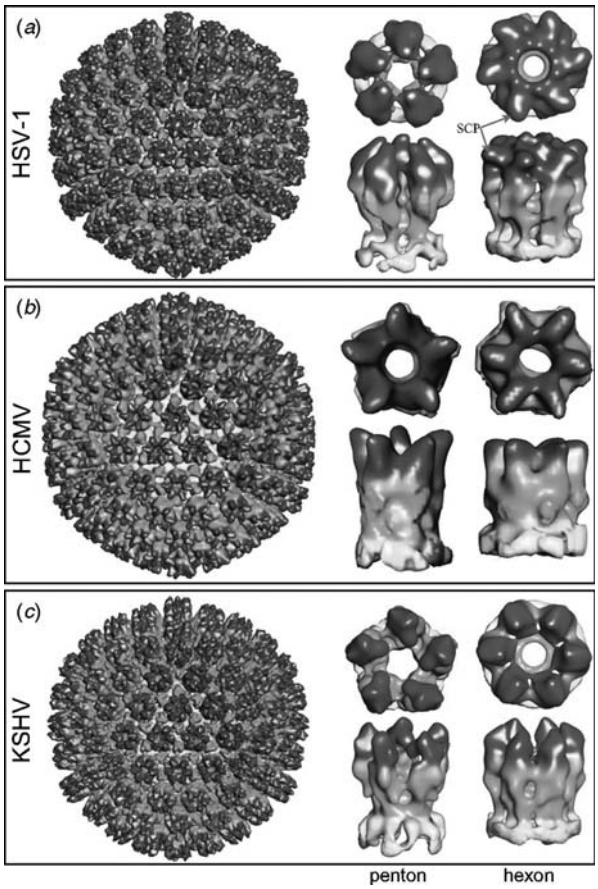

Fig. 3.4. Comparison of the three-dimensional structures of alpha, beta and gammaherpesvirus capsids. The capsid maps of HSV-1 (*a*), HCMV (*b*) and KSHV (*c*) are shown as shaded surfaces colored according to particle radius and viewed along an icosahedral three-fold axis. The resolution of the HSV-1 and KSHV capsid maps is 24 Å and that of the HCMV capsid (Butcher *et al.*, 1998) is 35 Å. The right two columns are detailed comparisons of a penton and an E hexon, which were extracted computationally from each map and shown in their top and side views. (See color plate section.)

capsid despite the similar molecular mass of its component proteins results in a greater center-to-center spacing of the capsomers compared to HSV-1 (Fig. 3.4(*b*)).

The structure of KSHV capsids was also determined by cryoEM to 24 Å resolution and exhibit structural features very similar to those of HSV-1 and HCMV capsids (Fig. 3.4(*c*)) (Trus *et al.*, 2001; Wu *et al.*, 2000). The KSHV and HSV-1 capsids are identical in size and capsomer organization. However, some notable differences are seen upon closer inspection. The KSHV capsid appears slightly more spherical than the HSV-1 capsid, which exhibits a somewhat angular, polyhedral shape. When viewed from the top, the hexons in the KSHV capsid appear flower-shaped, whereas those of HSV-1 have slightly tilted subunits and as a result appear more gear-shaped (see below). Also, the KSHV triplexes are slightly smaller and deviate less from threefold symmetry than the much-elongated triplexes in the HSV-1 capsid. The differences in the upper domains of HSV-1 and KSHV triplexes indicate that the HSV-1 triplexes are slightly taller. The radial density profiles show that the KSHV and HSV-1 capsids have identical inner radii of 460 Å (Wu *et al.*, 2000). Because both viruses also have similar genome sizes, their identical inner radii suggest that their DNA packing densities inside the capsids are similar. In contrast, betaherpesvirus capsids, such as those of HCMV, have a somewhat larger internal volume than HSV-1 or KSHV capsids (Bhella *et al.*, 2000; Chen *et al.*, 1999; Trus *et al.*, 1999). However, the increase in volume is disproportionate to the large increase in the size of the HCMV genome over the HSV-1 and KSHV genomes. This implies that the viral DNA is more densely packed into HCMV virions than into HSV-1 or KSHV virions.

In herpesvirus capsids, both the penton and hexon have a cylindrical shape (about 140-Å diameter, 160-Å height) with a central, axial channel approximately 25 Å in diameter (Fig. 3.4). The penton and hexon subunits both have an elongated shape with multiple domains, including upper, middle, lower, and floor domains. The middle domains of the subunits interact with the triplexes. The lower domains connect the subunits to each other and form the axial channels. While the upper domains of adjacent hexon subunits interact with one another, adjacent penton subunits are disconnected at their upper domains, resulting in the V-shaped side view of the pentons (Fig. 3.4). Another major difference between the penton and hexon concerns their floor domains. These domains play an essential role in maintaining capsid stability, as suggested by the higher-resolution structural studies of the HSV-1 capsid (Zhou *et al.*, 2000), where a long α-helix inserts into the floor domain of the adjacent subunit (Fig. 3.3(*b*)). The relative angle between the floor and lower domains is about 110° in the penton subunit and becomes less than 90° in the hexon subunit, making the penton to appear longer in its side view.

The HSV-1 penton and hexon subunits have the same basic shape as the HCMV and KSHV subunits (Fig. 3.4). Each consists of upper, middle, lower, and floor domains. However, the upper domains of the HSV-1 penton subunits point inward toward the channel, whereas those of the HCMV and KSHV penton subunits point outward. The upper domain of the KSHV subunit has a rectangular

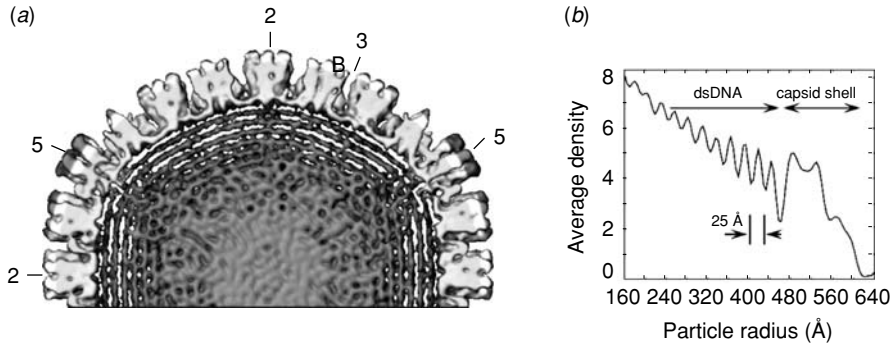

Fig. 3.5. Packing of dsDNA inside herpesvirus capsid (Yu *et al.*, 2003). (*a*) The upper half of a 100-Å thick central slice extracted from the 21 Å resolution reconstruction of the C-capsid of the rhesus rhadinovirus (RRV), a gammaherpesvirus and the closest KSHV homologue. The slice exhibits high-density features organized as multiple spherical shells inside the inner surface of the capsid floor. At least six concentric shells can be distinguished before the pattern becomes indistinct toward the center of the capsid. (*b*) Radial density distribution of the C-capsid obtained by spherically averaging the C-capsid reconstruction and plotted as a function of radius. It is evident that the distance between neighboring peaks is about 25 Å.

shape, while that of the HSV-1 penton subunit appears as a triangle. The most striking difference is that the HSV-1 hexon subunits contain an extra horn-shaped density which is not found in the HSV-1 penton (Fig. 3.4(*a*), arrow in right panel). This extra density binds to the top of each HSV-1 hexon subunit and has been shown to be the SCP, VP26, by difference imaging (Trus *et al.*, 1995; Zhou *et al.*, 1995), which associate with one another to form a hexameric ring around the hexon at a radius of approximately 600 Å. This accounts for the tilted or gear-like appearance of the HSV-1 hexon top view. The KSHV homolog of HSV-1 VP26 is ORF65. Difference map of anti-ORF65 antibody labeled and unlabeled KSHV capsids also showed that ORF65 binds only to the upper domain of the major capsid proteins in hexons but not to those in pentons (Lo *et al.*, 2003). The lack of horn-shaped densities on the hexons indicates that KSHV SCP exhibits substantially different structural features from HSV-1 SCP. The location of SCP at the outermost regions of the capsid suggests a possible role in mediating capsid interactions with the tegument and cytoskeleton proteins during infection.

Structure and packaging of viral genomic DNA

The sizes of the dsDNA genomes of different human herpesviruses vary substantially, e.g., the HCMV genome is 51% longer than HSV-1 (Davison *et al.*, 2003; McGeoch *et al.*, 2000). The major point of interest concerns the packing of their genomes within the capsids. The HCMV capsid is 117% larger than HSV-1. Besides the volume, factors such as DNA density, capsid capacity, and capsid expansion can also influence DNA packaging in viruses. The genome of HCMV might be more densely packed than that of HSV-1, or might induce expansion of the capsid upon packaging. Alternatively, the two viruses might have a similar capacity but differ in the amount of unoccupied space at the center of the capsids.

In HSV-1, the genomic DNA within the nucleocapsid is closely packed into multiple shells of regularly spaced densities, with 26 Å between adjacent DNA duplexes (Zhou *et al.*, 1999). The central slice and radial density plot in Fig. 3.5 indicate that the C-capsid of Rhesus rhadinovirus (RRV), a gammaherpesvirus, has an almost identical pattern of DNA organization to those observed in HSV-1, though slightly more compact, with a 25-Å inter-duplex distance (Yu *et al.*, 2003). Although the RRV capsid, like the KSHV capsid, has nearly the same diameter as the HSV-1 capsid (1250 Å), RRV has a slightly larger genome size than HSV-1, ~165 vs. 153 kb, respectively (Alexander *et al.*, 2000; Lagunoff and Ganem, 1997; Renne *et al.*, 1996; Searles *et al.*, 1999). Therefore, the smaller inter-duplex distance may merely reflect the need to compact this greater amount of DNA into the same volume within the capsid. HCMV has the largest genome (~230 kb) of all human herpesviruses but has a capsid that is only slightly larger (1300 Å diameter), and its DNA was shown to pack with an interduplex distance of only 23 Å (Bhella *et al.*, 2000). Based on the interduplex spacing and the genome sizes, we estimate that the closely packed DNA genomes of HSV-1, RRV, and HCMV would occupy a total volume of 3.52×10^8 Å^3, 3.51×10^8Å^3, and 4.05×10^8Å^3, respectively (Yu *et al.*, 2003). These volumes would measure approximately 92%, 92%, and 90% of the total available spaces inside the HSV-1, RRV, and

HCMV capsids, as estimated on the basis of their inner diameters of 900 Å, 900 Å and 950 Å, respectively. The 23–26 Å packing of strands of herpesvirus dsDNA is very close to the 20-Å diameter of B-type dsDNA, suggesting that herpesvirus genomes are packed as "naked" DNA without any bound histone-like basic proteins. In this regard, SDS-PAGE analyses demonstrated that the A-capsids and C-capsids have the same protein composition (Booy *et al.*, 1991; O'Connor *et al.*, 2003). In the absence of histone-like proteins, close packing of naked DNA would lead to a potentially strong electrostatic repulsion between the juxtaposed negatively charged DNA duplexes. This would make the packaging of DNA into procapsid energetically unfavorable, supporting the need for an energy-dependent DNA packaging machinery such as the bacteriophage-like connector recently reported in HSV-1 capsids (Newcomb *et al.*, 2001). Even so, it is conceivable that the negative charge of DNA may at least be partially neutralized by binding polyamines (Gibson and Roizman, 1971) or some other undiscovered small basic molecules to reduce the strong electrostatic repulsion.

Structure and assembly of tegument

Composition of viral tegument

The tegument occupies the space between the capsid and the envelope. Since the capsid and virion are ~125 nm and ~220 nm in diameter, respectively, the tegument represents a significant part of the virion space and indeed, contains approximately 40% of the herpesvirus virion protein mass (Gibson, 1996;). Since they are components of virions, tegument proteins are delivered to cells at the very initial stage of infection and they have the potential to function even before the viral genome is activated. Extensive studies, including amino acid sequencing and mass spectrometric analyses, have been carried out to determine the protein content of the tegument. These results have revealed the compositions of the teguments of HSV and HCMV, and provided insight into its function.

The tegument of HSV-1 contains more than 20 virus-encoded proteins (Roizman and Knipe 2001). The most notable proteins include the α-trans-inducing factor (αTIF, VP16), the virion host shutoff (vhs) protein (UL41), and a very large protein (VP1–2). VP16 functions as a transcription activator to induce the transcription of viral immediate–early genes, and in addition, plays an essential function as a structural component in the tegument (McKnight *et al.*, 1987; Preston *et al.*, 1988; Weinheimer *et al.*, 1992). The protein vhs is a non-sequence specific RNase that degrades most of the host mRNAs during the initial stage of viral infection, and facilitates the translation of viral mRNAs and viral gene expression (Everly *et al.*, 2002; Read and Frenkel, 1983). VP1–2 is found to be associated with a complex that binds to the terminal *a* sequence of the viral genome, which contains the signal for packaging the genome into the capsid (Chou and Roizman, 1989).

At least 30 virus-encoded proteins have been found in the HCMV tegument (Gibson, 1996; Mocarski and Courcelle, 2001). Significant progress has been made to delineate the function of these HCMV-encoded tegument proteins. For example, the UL69 protein acts to block cell cycle progression, while the UL99-encoded pp28 protein is required for cytoplasmic envelopment of the nucleocapsids (Hayashi *et al.*, 2000; Sanchez *et al.*, 2000; Silva *et al.*, 2003).

There are five predominant protein species found in the HCMV tegument: the high molecular weight protein (HMWP) encoded by UL48, the HMWP-binding protein encoded by UL47, the basic phosphoprotein (BPP or pp150) encoded by UL32, the upper matrix protein (UM or pp71) encoded by UL82, and the lower matrix protein (LM or pp83) encoded by UL83 (Gibson, 1996; Mocarski and Courcelle, 2001). Although their organization within the virion is not completely understood, these abundant proteins are believed to form the structural backbone of the tegument. UL48 and UL32 products, both of which are essential for viral replication (Dunn *et al.*, 2003; Meyer *et al.*, 1997), have been proposed to interact intimately with nucleocapsids (see below). Blocking UL32 expression resulted in accumulation of the nucleocapsid, suggesting that this protein is essential for tegument formation (Meyer *et al.*, 1997).

UL82 is also believed to be involved in direct interaction with the newly synthesized nucleocapsid, and is important for initiation of tegument assembly (Trus *et al.*, 1999). Moreover, the UL82-encoded pp71 protein is a transcriptional activator that helps to induce the transcription of the immediate-early genes within the infected cells (Liu and Stinski, 1992). UL83, the most abundant tegument protein, accounts for more than 15% of the virion protein mass (Gibson, 1996). The encoded pp65 protein has been reported to block major histocompatibility complex class I presentation of a viral immediate–early protein, and more recently, has been implicated to inhibit the induction of host interferon response (Browne and Shenk, 2003; Gilbert *et al.*, 1996). Remarkably, pp65 is not essential for viral replication and infectious virion production (Schmolke *et al.*, 1995). However, UL83 constitutes 90% of the protein mass in the noninfectious dense bodies, which have similar envelope structure, but lack a capsid core (Gibson, 1996). Non-infectious envelope particles, which contain B-capsid like core without the viral DNA genome, have a

reduced amount (30–60% lower) amount of pp65, as do low passage clinical isolates, compared to the laboratory-adapted AD169 and Towne strains (Gibson, 1996; Klages *et al.*, 1989). These observations suggest that UL83 serves as a nonstringent, volume-filling function in facilitating the assembly of virions, non-infectious enveloped particles, and dense bodies. Furthermore, the abundance of this protein in the viral particles and its function in blocking host immune response is believe to allow the virus to escape immune surveillance and significantly contributes to CMV survival (Browne and Shenk, 2003; Gilbert *et al.*, 1996).

Comparative structure of viral tegument

Overview of tegument structure

While significant progress has been made during the last few years to identify tegument proteins and study their functions, little is currently known about the structure of the tegument and the organization of the proteins within the tegument. Equally elusive is the pathway of the assembly and formation of the tegument, which involves the packaging of all the tegument proteins and is certainly a highly regulated, ordered process.

Recent electron cryomicroscopy studies on the virus-related particles of HSV-1, HCMV, and simian CMV (SCMV) provide significant insight into the structure and organization of the herpesvirus tegument (Chen *et al.*, 1999; Trus *et al.*, 1999; Zhou *et al.*, 1999). In these studies, the three-dimensional structures for the infectious virions or cytoplasmic tegumented capsids were reconstructed, and compared to the structures of the intranuclear capsids. The tegument can be seen in the virion as a region of relative low density covering an area in the 60–100 nm radius (Figs. 3.1(*a*), (*c*) and 3.6(*a*), (*c*)) (Chen *et al.*, 1999; Zhou *et al.*, 1999). Although the diameters of the nucleocapsids in different particles appear uniform, the sizes and shapes of the virus particles and the relative locations of nucleocapsids inside the particles vary. These observed variations suggest that most of the tegument proteins do not maintain rigid interactions with the enclosed nucleocapsids, and thus the bulk of the tegument layer does not possess icosahedral symmetry (Chen *et al.*, 1999; Zhou *et al.*, 1999).

The protein densities are also unevenly distributed across the tegument space. Studies on the localization of the tegument proteins have been reported using immunoelectron microscopy with antibodies specifically against tegument proteins and chemical treatment approaches for step-wise removal of layers of virion particles (Gibson, 1996; Steven and Spear, 1997). Several proteins have been found to be located at the tegument space distant to the nucleocapsids. For example, UL23 and UL24 are localized in the HCMV tegument space close to the inner side of the envelope membrane (Adair *et al.*, 2002).

Detailed comparison of the electron cryomicroscopic images of the intact virion particles, the cytoplasmic tegumented capsids, and the nucleocapsids, revealed the unique tegument densities that are present in virion and tegumented capsids but not in capsid preparations. Some of these tegument densities, which are closely associated with nucleocapsid, also exhibit a certain degree of symmetry, and their structures were reconstructed to a resolution of 18–30 Å (Fig. 3.6) (Chen *et al.*, 1999; Trus *et al.*, 1999; Zhou *et al.*, 1999). Since the surface of the nucleocapsid represents the starting site for tegument acquisition and envelopment, these tegument densities are believed to involve specific and direct interactions with capsid proteins and serve as anchors to recruit other tegument proteins for initiation of tegument formation. The tegument densities of HSV1 that are closely associated with the capsids exhibit a dramatic difference from those of HCMV and SCMV (Chen *et al.*, 1999; Trus *et al.*, 1999; Zhou *et al.*, 1999) (Fig. 3.6). This may not be unexpected since there is little evolutionary conservation in the sequence of tegument proteins between HSV-1 and CMV, and many CMV tegument proteins do not have sequence homologues in HSV-1 (Davison *et al.*, 2003; McGeoch *et al.*, 2000).

Tegument structure of HSV-1

Comparison of the maps of HSV-1 intact virion particles and B capsids revealed the marked differences between the two maps in the region of the pentons, which are highlighted in color in the superposition of the difference map on the B-capsid map (Fig. 3.6(*b*)). The most obvious difference is the presence of additional material extending from the surface of the pentons. The extra material has a molecular mass of 170–200 kDa, extends from the interface between the upper domains of two adjacent VP5 subunits in the penton and connects to the nearby triplexes that are made up of VP19C and VP23 proteins (Fig. 3.6(*b*)). The restriction of the tegument contacts to the pentons is consistent with previous observations of tightly attached tegument material at the vertices of capsids in negative stain and freeze-etching images of detergent-treated equine herpevirus virions (Vernon *et al.*, 1982). An identical pattern of tegument protein interaction was observed in a VP26-minus virion mutant (Chen *et al.*, 2001). This result indicates that the lack of tegument association of the HSV-1 hexons is not due to the presence of VP26 on the hexon upper domain, but rather likely due to the inherent structural difference on the upper domains of penton and hexon VP5.

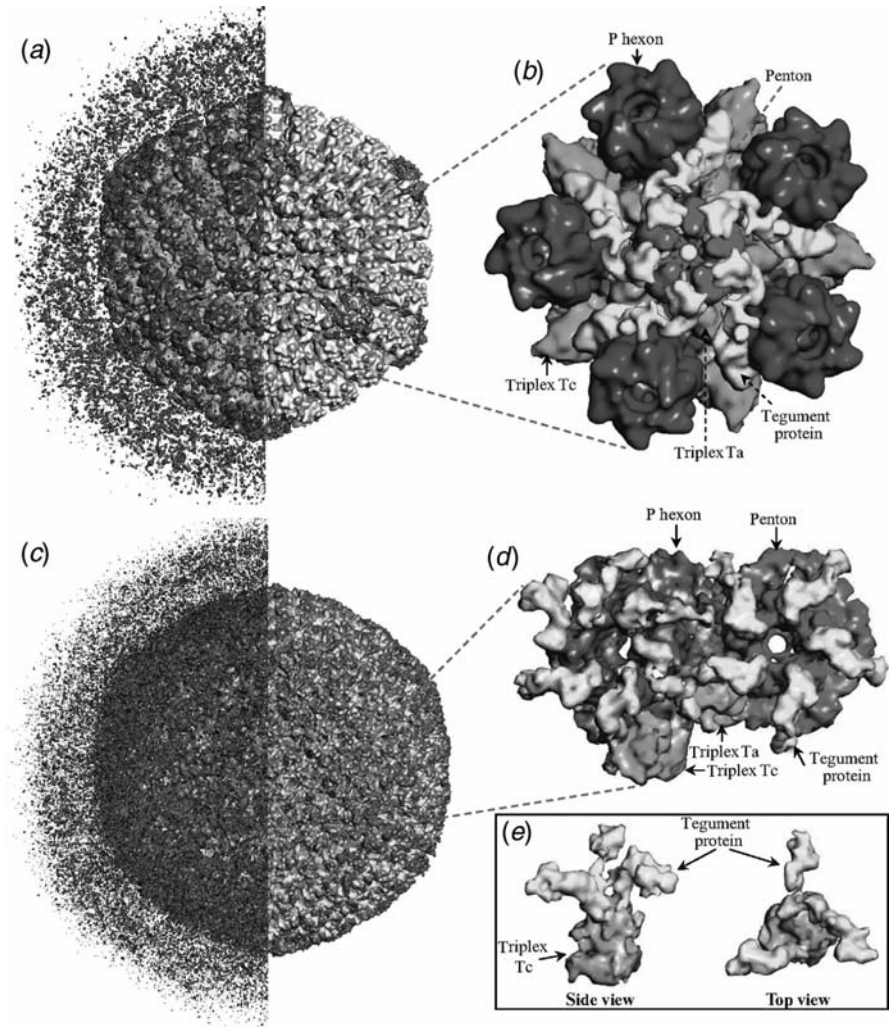

Fig. 3.6. Difference of the anchored tegument proteins between HSV-1 ((*a*) and (*b*)) and HCMV ((*c*)–(*e*)). ((*a*) and (*c*)) Radially color-coded shaded surface views of the three-dimensional reconstruction of HSV-1 (*a*) and HCMV (*c*) virions as viewed along an icosahedral three-fold axis. The bulk of the tegument components and the viral envelope are not icosahedrally ordered or polymorphic, thus appearing as disconnected low densities in the icosahedral reconstruction. These disconnected densities were masked out for the right hemisphere to better reveal the icosahedrally ordered tegument proteins, which are shown in blue to purple colors in (*a*) and in purple in (*c*). ((*b*) and (*d*)) Close-up views of the region indicated in (*a*) and (*c*), respectively, showing the molecular interactions of the tegument proteins (yellow) with the penton (red), P hexon (blue) and triplexes (green). In HSV-1, contrary to the extensive tegument association with all hexons, the tegument densities do not interact with any hexon. (*e*) Extracted triplex HCMV Tc with its attached tegument densities. Three tegument densities interact with the upper domain of each triplex (insert Table 3.1). (See color plate section.)

Based on its close association with the capsid and relative abundance in the tegument, the essential tegument protein VP1–3 has been proposed to constitute a major part of the protein complexes representing the tegument material (Zhou *et al.*, 1999). VP1–3 is an interesting yet poorly characterized protein. It has been shown that VP1–3 is associated with a complex that binds to the terminal *a* sequence of the viral genome, which contains the signal for genome packaging into the capsid (Chou and Roizman, 1989). A temperature-sensitive mutant (*ts* B7) with a mutation in VP1–3 fails to release viral DNA from the infecting capsids into the nucleus during viral decoating process (Batterson and Roizman, 1983). Since the penton has been suggested to be the route by which viral DNA leaves the capsid (Newcomb and Brown, 1994), an interaction between VP1–3 and the penton proteins would place it in an appropriate

position to influence the passage of the viral genome. Further studies are needed to test these hypotheses and completely reveal the identity of the proteins coding for the tegument material.

Tegument structure of CMV

The tegument densities of HCMV that are closely associated with nucleocapsid are dramatically different from those of HSV-1 (*cf.* Fig. 3.6 (*a*) and (*c*)) (Chen *et al.*, 1999). A difference map between the HCMV particles and B-capsids revealed a thin shell of loosely connected filamentous densities, representing the icosahedrally ordered, capsid-proximal portion of the tegument in HCMV (Fig. 3.6(*c*)–(*e*)). Unlike HSV-1, the tegument densities interact with all of the structural components of the nucleocapsid: penton (made up of major capsid protein UL86), hexon (consisted of UL86 and smallest capsid protein UL48.5), and triplex (composed of minor capsid protein UL85 and its binding protein UL46). Figure 3.6(*d*) shows the close-up views of a region from the intact virus reconstruction that includes one penton (red), one P hexon (blue), and two representative adjacent triplexes Ta and Tc (green). Superimposed on the penton and hexon are their associated tegument densities (yellow). Clusters of five and six tegument densities attach to the pentons and hexons, respectively. Moreover, neighboring clusters associate with each by bridging over the intercapsomer space, apparently using triplexes as piers. Each of the filamentous tegument densities, which is about 12 nm in length and 2–3 nm in diameter, acts as the bridge arch. Thus, the capsid appears to act as the scaffold of the ordered tegument protein layer. These results imply that the ordered tegument layer cannot form without the underlying capsid and are consistent with the observations that no such tegument layer was found in dense bodies (Chen *et al.*, 1999).

Detailed examination of the interactions between triplexes and tegument densities further revealed minor differences between the structures of SCMV cytoplasmic tegumented capsids and HCMV particles (Chen *et al.*, 1999; Trus *et al.*, 1999). In SCMV cytoplasmic capsids, two tegument densities were found to be associated with each triplex. In contrast, three densities were shown to be attached to each triplex of the nucleocapsid of the HCMV particles (Fig. 3.6(*e*)). It is conceivable that the extra tegument densities observed in HCMV structure may represent those that were loosely associated with the capsids and probably lost during the purification of the SCMV capsids.

Based on their relative abundance and close association with the nucleocapsids, two CMV tegument proteins, UL32 and UL82, have been proposed to constitute the majority of the observed tegument material that attach to the capsids (Chen *et al.*, 1999; Trus *et al.*, 1999). UL82, which encodes a transcriptional activator (Liu and Stinski, 1992), has a molecular weight of ~70 kDa, similar to the estimated molecule mass of the capsomer-capping tegument protein densities. UL32 has been suggested to be involved in the transport of DNA-containing capsids through nuclear membrane during envelopment or in the stabilization of capsids in the cytoplasm (Meyer *et al.*, 1997). In recent experiments, CMV virion particles were subjected to different chemical conditions, which do not disrupt the integrity of the nucleocapsids, to selectively remove the components not tightly associated with the capsids. These experiments showed that most of the known tegument proteins, including UL99 and UL83, are removed, but UL32 is not affected (Yu, X., Lee, M., Lo, P., Liu, F., and Zhou, Z. H., unpublished results). Thus, these results further suggest that UL99 and UL83 are loosely and distantly associated with capsids and that UL32 is in close proximity and possibly involved in direct interactions with the capsids.

Structure and assembly of viral envelope

The envelope contains most, if not all, of the virion glycoproteins. Each of the herpesviruses encodes a set of 20–80 glycoproteins, very few of which are highly conserved among all the herpesviruses (Kieff and Rickinson, 2001; Mocarski and Courcelle, 2001; Roizman and Knipe, 2001). For example, HSV-1 encodes at least 20 glycoproteins, 11 of which are found in the virions (Roizman and Knipe, 2001). HCMV potentially encodes more than 75 membrane-associated proteins, at least 15 of which are found in the virions (Mocarski and Courcelle, 2001). The exact organization of viral surface glycoproteins in the envelope is not completely understood. Virion glycoproteins are found to aggregate into complexes on the surface of the virion. For example, HCMV glycoproteins gH, gL, and gO are associated to form a heterotrimeric envelope glycoprotein complex (Gibson, 1996; Mocarski and Courcelle, 2001). These proteins may form their complexes in the cellular membrane compartment before trafficking to the viral envelope. However, it remains possible that further higher-order complexes are assembled after these protein components are delivered in the viral envelope membrane.

In addition to the viral encoded glycoproteins, the envelope also contains numerous host proteins or constituents. For example, host proteins associated with HCMV envelope include β2-microglobumin, CD55 and CD59, and annexin II (Grundy *et al.*, 1987a, b; Wright *et al.*, 1995). These molecules may participate in the induction of host cellular responses. It is conceivable that these host proteins, in combination with the viral encoded G protein-coupled

receptors associated with the HCMV envelop, play an important role in modulating host cell response during initial virus attachment, as observed in recent studies (Compton *et al.*, 2003; Zhu *et al.*, 1998).

Herpesvirus envelopment is believed to take place initially at the inner nuclear membrane, and then further proceeds with an envelopment/de-envelopment process that allows the capsid to cross the double nuclear membranes and other cytoplasmic membrane structures (Gibson, 1996; Steven and Spear, 1997). Cytoplasmic envelopment of HSV-1 and CMV capsids can also take place in endosomes as well as Golgi networks (Eggers *et al.*, 1992). Thus, it is not surprising that the envelope contains diverse lipid components that are associated with different parts of the cytoplasmic membrane system in addition to the nuclear membrane. These components include the phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol (Roby and Gibson, 1986). Whether these lipid components may be important in maintaining the integrity of the viral envelope has not been determined and their functional role in stabilizing virion structure is unknown.

The envelopment process appears to be not affected in the absence of a mature capsid since noninfectious enveloped particles and dense bodies can be produced and contain similar envelop contents. In the case of HCMV, pp65 constitutes at least 90% of the dense body protein mass (Gibson, 1996). It is conceivable that this protein may contain signals that promote its own envelopment. There are presumably interactions between proteins of the tegument and envelope that promote the envelopment process. Indeed, recent results indicate that HCMV UL99, a tegument protein, facilitates the cytoplasmic envelopment of the nucleocapsid (Sanchez *et al.*, 2000; Silva *et al.*, 2003). Two HSV-1 membrane proteins, UL34 and UL31, are also implicated to be essential for viral envelopment (Reynolds *et al.*, 2001). Further studies on the organization of the proteins in these particles and their potential interactions with envelope components will provide insight into the process of their assembly.

Other constituents in the virions

Recent studies indicated that viral mRNAs were found in HSV-1 and HCMV virions (Bresnahan and Shenk, 2000; Sciortino *et al.*, 2001). These mRNAs appear to be packaged selectively into the infectious virion particles. They have been proposed to function to facilitate the initiation of viral infection upon viral entry. It is unknown whether these virion mRNAs play a role in maintaining the integrity of the virion structure, as ribosomal RNAs provide the backbone for ribosome assembly.

Depending on the approach of how the virions are prepared and the quality of the preparations being analyzed, numerous host constituents, including lipids, polyamines, and cellular enzyme and structural proteins, are also found to be associated with the viral particles. In particular, two of these host constituents, polyamines and actin-related protein (ARP), may play an important role in stabilizing and maintaining the intact structure of the infectious particles. Two kinds of polyamines, spermidine and spermine, have commonly been found in herpesvirus virions, including HSV-1 and HCMV (Gibson and Roizman, 1971; Gibson *et al.*, 1984). In highly purified HSV-1 virion preparations, there are about 70 000 molecules of spermidine and 40 000 molecules of spermine per virion (Gibson and Roizman, 1971). The functions of these polyamines are believed to provide positive charges to neutralize the highly negatively charged viral DNA genome during the genome replication and packaging. This hypothesis is consistent with the observations that none of the herpesvirus capsid proteins are highly positive charged and addition of arginine facilitates capsid assembly and virion production (Mark and Kaplan, 1971). Spermidine appears to be in the tegument while spermine is localized in the nucleocapsid. It is estimated that the spermine contained in the virion has the capacity to neutralize about 40% of the DNA phosphate, consistent with its role in stabilizing the packed genomic DNA in the nucleocapsid core.

In analyzing highly purified HCMV virions as well as noninfectious enveloped particles and dense bodies, Baldick and Shenk first reported the presence of a substantial amount of a cellular actin-related protein (ARP) in the tegument compartment (Baldick and Shenk, 1996). The exact localization of the ARP is currently unknown, and preliminary studies using stepwise chemical treatment of HCMV virion for removal of different parts of the particles have suggested that ARP is localized in the tegument space distant from the nucleocapsid (Yu, X., Lee, M., Lo, P., Liu, F., and Zhou, Z. H., unpublished results). Based on their roles for providing cytoskeleton and maintaining cellular structure and morphology, it is conceivable that actin-related proteins stabilize the tegument structure. Meanwhile, some ARPs have been implicated in participating dynein-driven microtubule transport system (Lees-Miller *et al.*, 1992; Schroer *et al.*, 1994). Given the fact that viral capsid trafficking from cytoplasm to the nuclear pore complex is driven by the dynein-microtubule system (Dohner *et al.*, 2002; Sodeik *et al.*, 1997), it is possible that these ARPs are specifically incorporated into the teguments and facilitate the transport of the viral particles from the nucleus to

the cytoplasmic membrane during viral envelopment and to the nucleus during post-penetration. Further studies are needed to completely elucidate the function of these proteins in assembly and maintenance of the virus structure.

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4

Comparative analysis of herpesvirus-common proteins

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Introduction

Despite the evolutionary and biological divergence represented by the nine human herpesviruses that have been classified into three broad subgroups, a large number of herpesvirus-common (core) gene products are evolutionarily conserved (Table 4.1 see chapter 2). These appear to carry out functions upon which every herpesvirus relies because all exhibit a common virion structure, a core genome replication process, and similar entry and egress pathways. These herpesvirus common functions are most often recognized through deduced protein sequence similarity that extends throughout alpha-, beta-, and gammaherpesviruses subfamilies infecting mammals, reptiles and birds (see Chapter 2, Table 2.2). These herpesviruses exhibit conservation that suggests a shared common ancestor at least 50 million years ago. Other evolutionarily distant herpesviruses infecting fish, amphibians, and invertebrates share less similarity with these better-studied herpesviruses, suggesting a common evolutionary origin dating back over 150 million years. In the more distant relatives, a common virion structure, genome organization and similarity across a small subset herpesvirus-common gene products provide the evidence of a common origin.

A few herpesvirus-common gene products have been recognized via a common enzymatic or binding activity long before any systematic genome sequence analysis became available. The homologous function of envelope glycoprotein B, DNA polymerase, alkaline exonuclease and single strand DNA binding protein, to give a few examples, emerged from biochemical studies in a number of herpesvirus systems. Given the high level of conservation and the importance of DNA synthesis as a target for antiviral inhibitors, these remain among the most broadly studied and best understood of the core gene products. DNA synthesis functions and virion structural components are also among the most highly conserved based on sequence comparisons. Importantly, however, common activity of well-recognized core functions, such as the DNA polymerase processivity factor, the smallest capsid protein or a capsid triplex component, is not based on the level of primary amino acid sequence identity, but rather is supported by a common role in several herpesviruses. Additional core functions initially recognized based on activity have undergone evolutionary divergence to take on new functions. For example, the large subunit of ribonucleotide reductase (HSV-1 *UL39* gene product), which associates with a small subunit to form an active enzyme in the alphaherpesviruses and gammaherpesviruses, is expressed without small subunit in the betaherpesviruses (HCMV, HHV-6, HHV-7) and completely lacks enzymatic activity. This leaves a question as to its true role. Thus, herpesvirus-common proteins may preserve common function as well as sequence homology, function with only limited sequence homology, or sequence homology with distinct function. Homologs may therefore be predicted to carry out similar functions in most situations, but will certainly not behave identically in all settings.

Herpesvirus-common gene products have been recognized as key proteins that form the characteristic herpesvirus virion structure and provide key common functions for the replicative cycle, beginning with entry into cells, continuing through the process of viral DNA synthesis and nucleic acid metabolism and concluding with capsid maturation and egress of virions. The presence of herpesvirus-common genes (see Chapter 2) allows predictions about functional conservation; however, most functional information has been derived from studies in a single or at most two herpesvirus subfamilies. The requirement for herpesvirus-common functions varies considerably with cell type, as well as between viruses of the same

Table 4.1. Identity, function and proposed nomenclature for known and putative functions of herpesvirus-common gene products of human herpesviruses

Common name[a]	Abbrev. name	HSV	VZV	HCMV	HHV 6/7	EBV	KSHV	Key function
Capsid								
major **c**apsid **p**rotein[c]	MCP	UL19	40	UL86	U57	BcLF1	ORF25	hexon, penton, capsid structure
triplex monomer[c]	TRI1	UL38	20	UL46	U29	BORF1	ORF62	TRI1 and TRI2 assoc to form TRI complex, capsid structure
triplex dimer[c]	TRI2	UL18	41	UL85	U56	BDLF1	ORF26	
small **c**apsid **p**rotein[b]	SCP	UL35	23	UL48A	U32	BFRF3	ORF65	capsid transport
portal protein	PORT	UL6	54	UL104	U76	BBRF1	ORF43	penton for DNA encapsidation
portal **c**apping **p**rotein[c]	PCP	UL25	34	UL77	U50	BVRF1	ORF19	covers portal in mature virions
Tegument and cytoplasmic egress								
virion **p**rotein **k**inase	VPK	UL13	47	UL97	U69	BGLF4	ORF36	phosphorylation, regulation
largest **t**egument **p**rotein[c]	LTP	UL36	22	UL48	U31	BPLF1	ORF64	uncoating, secondary envelopment
LTP binding **p**rotein[b]	LTPbp	UL37	21	UL47	U30	BOLF1	ORF63	
encapsidation and **e**gress **p**rotein[c]	EEP	UL7	53	UL103	U75	BBRF2	ORF42	nuclear egress
cytoplamsic **e**gress **t**egument **p**rotein[b]	CETP	UL11	49	UL99	U71	BBLF1	ORF38	secondary envelopment, cytoplasmic egress
CETP binding **p**rotein[b]	CETPbp	UL16	44	UL94	U65	BGLF2	ORF33	
cytoplasmic **e**gress **f**acilitator-**1**[b]	CEF1	UL51	7	UL71	U44	BSRF1	ORF55	cytoplasmic egress
encapsidation **c**haperone **p**rotein[b]	ECP	UL14	46	UL95	U67	BGLF3	ORF34	TERbp chaperone
capsid **t**ransport **t**egument **p**rotein[c]	CTTP	UL17	43	UL93	U64	BGLF1	ORF32	capsid transport in the nucleus
cytoplasmic **e**gress **f**acilitator-**2**[b]	CEF2	UL21	38	UL88	U59	BTRF1	ORF23	egress, interact with CETPbp
		UL24	35	UL76	U49	BXRF1	ORF20	putative membrane or tegument
Envelope								
glycoprotein **B**[c]	gB	UL27	31	UL55	U39	BALF4	ORF8	heparan-binding, fusion
glycoprotein **H**[c]	gH	UL22	60	UL75	U48	BXLF2	ORF22	gH assoc, fusion
glycoprotein **L**[c]	gL	UL1	37	UL115	U82	BKRF2	ORF47	gL assoc, fusion
glycoprotein **M**[b]	gM	UL10	50	UL100	U72	BBRF3	ORF39	gN assoc
glycoprotein **N**[b]	gN	UL49A	9A	UL73	U46	BLRF1	ORF53	gM assoc
Regulation								
multifunctional **r**egulator of **e**xpression[c]	MRE	UL54	4	UL69	U42	BSLF1 BMLF1	ORF57	transcriptional, RNA transport regulation
DNA Replication, recombination and metabolism								
DNA **pol**ymerase[c]	POL	UL30	28	UL54	U38	BALF5	ORF9	DNA synthesis
DNA **p**olymerase **p**rocessivity **s**ubunit[c]	PPS	UL42	16	UL44	U27	BMRF1	ORF59	POL processivity
helicase-**p**rimase **A**TPase subunit[c]	HP1	UL5	55	UL105	U77	BBLF4	ORF44	HP1, HP2 and HP3 assoc to form HP, unwinding and primer synthesis
helicase-**p**rimase RNA pol subunit **B**[c]	HP2	UL52	6	UL70	U43	BSLF1	ORF56	
helicase-primase subunit **C**[c]	HP3	UL8	52	UL102	U74	BBLF2 BBLF3	ORF40 ORF41	
single **s**trand DNA **b**inding protein[c]	SSB	UL29	29	UL57	U41	BALF2	ORF6	DNA fork, recombination
alkaline deoxyribonuclease[b]	NUC	UL12	48	UL98	U70	BGLF5	ORF37	recombination
deoxyuridine triphosphatase[b]	dUTPase	UL50	8	UL72	U45	BLLF3	ORF54	reduce dUTP
uracil-DNA glycosidase[b]	UNG	UL2	59	UL114	U81	BKRF3	ORF46	remove uracil from DNA
ribonucleotide **r**eductase large subunit[b]	RR1	UL39	19	UL45	U28	BORF2	ORF61	active enzyme only in viruses with RR2
Capsid assembly, DNA encapsidation and nuclear egress								
maturational **pr**otease[c]	PR	UL26	33	UL80	U53	BVRF2	ORF17	capsid assembly, scaffolding, DNA encapsidation
assembly **p**rotein[c]	AP (NP)[d]	UL26.5 (UL26)	33.5 (33)	UL80.5 (UL80)	U53.5 (U53)	BdRF1 (BVRF2)	ORF17.5 (ORF17)	

(*cont.*)

Table 4.1. (*cont.*)

Common name[a]	Abbrev. name[a]	HSV	VZV	HCMV	HHV 6/7	EBV	KSHV	Key function
capsid **t**ransport **n**uclear **p**rotein[c]	CTNP	UL32	26	UL52	U36	BFLF1	ORF68	capsid transport to sites of DNA replication
terminase **A**TPase subunit 1[c]	TER1	UL15	42	UL89	U66	BGRF1	ORF29	TER1 and TER2 form TER, packaging machinery
			45			BDRF1		
terminase DNA binding subunit 2[c]	TER2	UL28	30	UL56	U40	BALF3	ORF7	
terminase **b**inding **p**rotein[c]	TERbp	UL33	25	UL51	U35	BFRF1A	ORF67	TER assoc
nuclear **e**gress **m**embrane **p**rotein[c]	NEMP	UL34	24	UL50	U34	BFRF2	ORF66	nuclear egress, primary envelopment
nuclear **e**gress **l**amina **p**rotein[c]	NELP	UL31	27	UL53	U37	BFLF2	ORF69	

[a] proposed.
[b] required for replication in some viruses or some settings.
[c] required for replication in all viruses and settings tested.
[d] AP and NP are related proteins derived from different primary translation products.

or different subfamilies. Currently, 36 of the 40 core functions have an impact on replication in at least one herpesvirus and in at least some experimental setting (Table 4.1), although many are not absolutely essential for replication in any of the herpesviruses where they have been studied. The striking cell type dependence of so many herpesvirus functions suggests that many of these proteins carry out activities that are redundant with other viral or cellular functions. This chapter will seek to deduce the general role of each core function based on data available in different systems. The phenotype of mutants showing even a modest growth defect under any conditions will be an important component for consideration. All core functions are likely to be important for viral replication and pathogenesis in the host even when considered dispensable for replication in cultured cells. While even limited information is useful, experimental confirmation across several distinct viruses builds confidence in the common role of homologs, but this information is limited in many cases.

All herpesviruses form particles that have a characteristic 125–130 nm icosohedral capsid containing a linear DNA genome, surrounded by a protein-containing tegument enclosed in a host-membrane derived lipid bilayer envelope modified by virus-encoded glycoproteins. The overall virion particle size ranges from 200 to 300 nm, depending on the particular virus. This common structure provides the strongest evidence for evolutionary conservation in the replication and maturation processes. The set of core, homologous open reading frames (ORFs) are predominantly involved in the processes of DNA replication through maturation and egress from cells (Fig. 4.1). These also facilitate estimations of the evolutionary relatedness of the three major subfamilies (alpha-, beta-, and gammaherpesviruses) comprising mammalian and avian herpesviruses (see Chapter 2). Recognizable sequence homologs of only three of these proteins (DNA polymerase, DUT and a terminase subunit are conserved in the genomes of every known herpesviruses, including those infecting amphibians, fish, and invertebrates. Of these, the gene for the terminase subunit is remarkable, producing a conserved spliced mRNA that remains a key genomic feature (Davison, 2002) of every herpesvirus genome that has been annotated. The nine distinct human herpesviruses, including three members of alphaherpesviruses (HSV-1, HSV-2 and VZV), four members of the betaherpesviruses (CMV, HHV-6A, HHV-6B and HHV-7) and two members of the gammaherpesviruses (EBV and KSHV/HHV-8) share 40 of the 43 ORFs that have been included in the core set (see Chapter 2 Table 2.2). Although widely distributed amongst alpha- and gammaherpesviruses characterized to date, a thymidine kinase (TK) and a small subunit of ribonuclease (RR2) are absent from all betaherpesviruses. Although all herpesviruses appear to use sequence-specific DNA binding proteins to initiate DNA replication, a homolog of DNA replication origin binding protein (OBP) is not conserved in cytomegaloviruses or gammaherpesviruses.

This chapter stresses common features, while evolution has clearly provided the herpesviruses with a broad canvas on which to evolve the remarkable range of biological properties that characterize individual members. Genome annotation and comparison has suggested a higher level of similarity within subfamilies than between biologically distinct subfamilies. Even though biological properties of the individual subfamily members are distinct, they retain a recognizable evolutionary link.

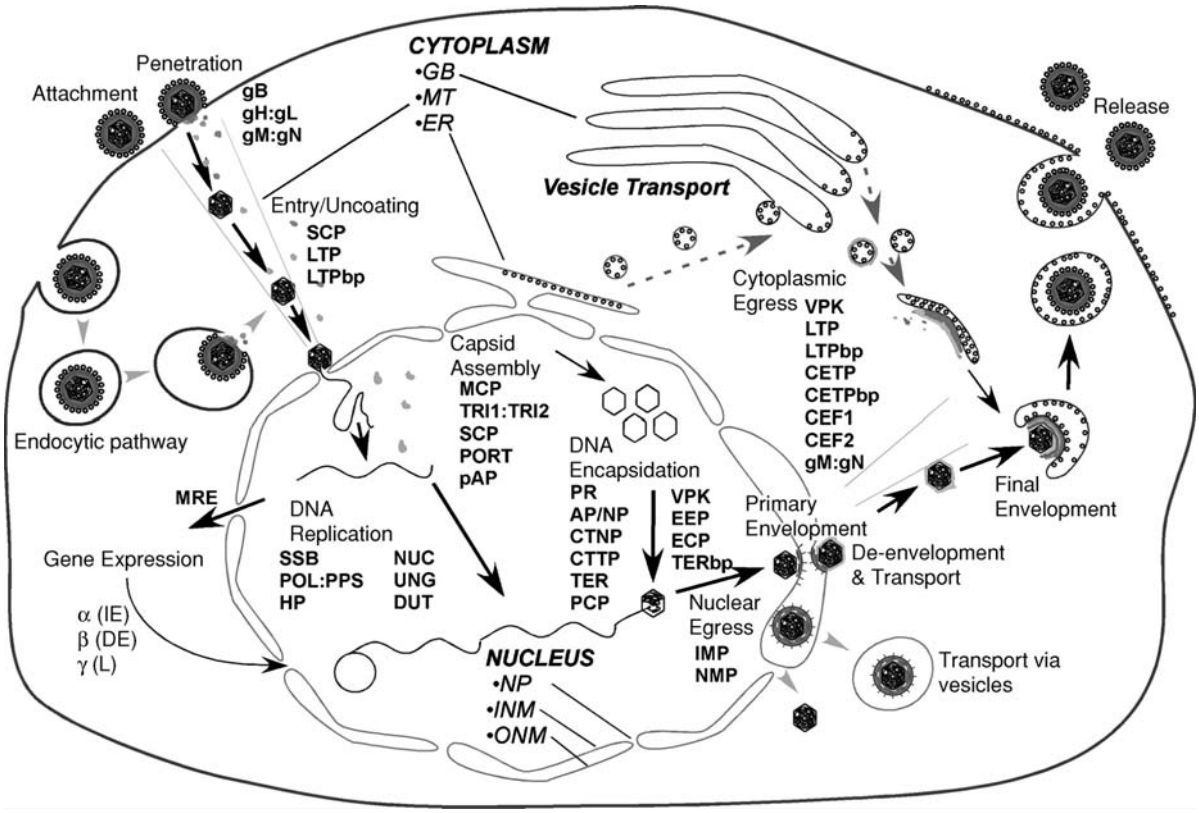

Fig. 4.1. Summary of replication functions carried out by herpesvirus-conserved gene products. Major steps in productive replication are indicated in larger font style and core functions contributing to each step are listed by their proposed abbreviated designations (see Table 4.1). The major entry pathway (black arrows) employs direct fusion at the cell surface (attachment and penetration), which is dependent upon gB and the gH:gL complex, followed by nucleocapsid transport along microtubules to nuclear pores where viral DNA is released into the nucleus. Alternatively, in certain cell types, entry follows the endocytic pathway and virion fusion with an endocytic vesicle (grey arrowheads). Uncoating requires the envelope fusion machinery (gB, gH:gL and in some cases gM:gN) as well as the LTP:LTPbp to direct docking at nuclear pores and release of virion DNA into the nucleus. Following entry and uncoating, one core regulatory protein (MRE) is involved in transcriptional and post-transcriptional regulation. DNA replication depends on several core replication fork proteins (SSB, POL:PPS, HP) as well as accessory functions (NUC, UNG, DUT). Capsid assembly uses MCP, TRI1:TRI2, SCP, and PORT. Pre-formed capsids translocate to sites of DNA replication where PRO, AP/NP (AP and NP are related proteins), CTNP, TER, TERbp and PCP, with possible accessory functions EEP, ECP and CTTP complete the encapsidation of viral DNA. Nuclear egress is controlled by NEMP and NELP. The main pathway of cytoplasmic egress (black arrows) and secondary (final) envelopment is controlled VPK, LTP:LTPbp, CETP, and CETPbp, with possible accessory proteins CEF1, CEF2 and gM:gN. Nucleocapsids are transported on MT and virion envelope glycoproteins follow vesicle transport to sites of final envelopment. Alternative maturation pathways of vesicle formation at the outer nuclear membrane with the mature virion following vesicle transport pathways or release of nucleocapsids directly through nuclear pores into the cytoplasm for transport to sites of final envelopment remain possible (grey arrowheads). Golgi body (GB), microtubules (MT) and endoplasmic reticulum (ER) are identified in the cytoplasm, and nuclear pores (NP), inner nuclear membrane (INM) and outer nuclear membrane (ONM) are identified in the nucleus. The cellular vesicle transport pathway from the ER to GB is also designated (dashed grey arrows).

There is little doubt that herpesviruses encode additional structurally related, functionally similar proteins that are not recognized as sequence homologs and all herpesvirus-common components cannot be recognized through sequence information alone. Other structural and functional properties complement sequence information and may constitute an independent set of criteria on which comparisons can be based. Relative position in a cluster of conserved ORFs, biological activity and the phenotype of mutant viruses all provide important comparative information. The existence of ORFs that fail to show sequence identity but are included in the list of core proteins due to

other information, such as the functional information on DNA polymerase processivity subunit already mentioned, suggests future work will require consideration of a broader set of characteristics. Additional structural and functional homologs will likely emerge as viral proteins are studied in greater detail by X-ray diffraction as by well as computer programs that model primary amino acid sequence on known protein structures.

Available information on the activities of core functions has often been generated in only one of the nine human herpesviruses, sometimes using herpesviruses of veterinary interest (e.g., PRV) and sometimes using rodent herpesviruses (e.g., MCMV). The remarkable diversity in functional organization, replication, latency and disease patterns exhibited by diverse human herpesviruses contrast the common activities of core functions which are key to viral replication. Functions will therefore be presented in relationship to the virus replicative cycle, starting with virion structure and entry, proceeding through regulation of gene expression, DNA synthesis, processing, and packaging and, finally, maturation and egress. Some of the functions involved in entry are structural components that are also involved with maturation and release of progeny virus, but most of the core functions can be implicated in at least one step in the replication pathway, and this is sometimes dependent on cell type. These core replication processes, including entry into cells and viral DNA synthesis, as well as the overall scheme of assembly, maturation and egress occur via evolutionarily conserved proteins and mechanisms. The role of core functions is best understood where they have been subjected to study by a combination of genetics and cell biology, this is often in alphaherpesviruses such as HSV-1 and PRV or in betaherpesviruses such as HCMV and MCMV. There have been many reviews dealing with herpesvirus-common features and evolutionarily common themes that have emerged over the years, both from a biological perspective (Roizman, 1999; Roizman and Pellett, 2001) as well as from a variety of analyses derived from genomic sequence (Davison, 2002; Karlin *et al.*, 1994; McGeoch *et al.*, 2000).

There are several common genome features described in Chapter 2 beyond the ultrastructural appearance of capsids and core proteins described in Chapter 3. All herpesviruses have a linear DNA genome that is cleaved from concatemers formed during replication, which where known, uses a conserved recognition sequence and leaves single base 3 overhanging nucleotide at each genomic terminus (Mocarski and Roizman, 1982). The position of origins of DNA replication that control DNA synthesis during the replicative cycle is conserved in most herpesviruses with a common location adjacent to the conserved single stranded DNA binding protein gene. While exceptions exist, the common evolutionary origin of these viruses is very clear from the range of conserved *cis*-acting elements as well as proteins.

Virion structural proteins

The virion particle of herpesviruses consists of a DNA-containing nucleocapsid with 162 regularly arranged capsomeres arranged in a $T = 16$ icosohedral lattice forming the protein shell. Detailed structural information has been derived from cryo-electron micrograph (cryo-EM) reconstructions involving studies on several different herpesviruses (Chapter 3). In the virion, the nucleocapsid is covered by a protein matrix or tegument that is surrounded by a lipid bilayer envelope derived from host cell membranes into which at least a dozen viral envelope proteins are inserted. Human herpesviruses have been estimated to have as few as 37 (e.g., alphaherpesviruses) and to well over 50 (e.g., cytomegaloviruses) virion proteins (Bortz *et al.*, 2003; Johannsen *et al.*, 2004; Kattenhorn *et al.*, 2004; Varnum *et al.*, 2004; Zhu *et al.*, 2005). About half (22) of the herpesvirus-conserved proteins are components of the virion, providing a genetic basis for the common ultrastructural appearance of all herpesviruses. Additional core proteins collaborate with structural proteins during maturation and egress (see below). Although similar in size, herpesvirus nucleocapsids package double-stranded DNA genomes range from a low of 125 kilobase pairs (VZV) to a high of 240 kilobase pairs (CCMV), which is remarkable given such a uniform capsid shell. The virion provides protection of the viral genome during transmission and mediates a two-stage entry process, first a fusion event between the envelope and cellular membranes that leads to release of the nucleocapsid into the cytoplasm and second a trafficking event that delivers the viral nucleocapsid to the nucleus where the genome is released.

Icosohedral herpesvirus capsids are composed of five herpesvirus-conserved proteins, the major capsid protein (MCP, HSV-1 *UL19* gene product), triplex monomer and dimer proteins (TRI1 and TRI2, HSV-1 *UL38* and *UL18* gene products, respectively), the smallest capsid protein (SCP, HSV-1 *UL35* gene product) and the portal protein (PORT, HSV-1 *UL6* gene product). The 150 hexons that make up the bulk of the capsid consist of six MCP molecules together with six molecules of SCP. Eleven of the 12 capsid pentons consist of five MCP molecules without SCP. One specialized penton consists of 12 molecules of PORT and has an axial channel for entry and exit of viral

DNA. The 125–130 nm diameter capsid has a wall that is 15 nm thick. Although different in shape, each hexon and penton appears cylindrical. Based on cryo-EM studies of HSV-1, HCMV, and KSHV (Chapter 3), the hexons and pentons are held in place by interactions between their bases on the inner side as well as by interconnections via triplexes located midway through the capsid shell. Detailed analyses suggests that beta- and gammaherpesvirus capsids resemble one another more closely than either resembles alphaherpesvirus capsids. Although the least conserved of the structural proteins, SCP is located at hexon tips, and absent from penton tips, in all herpesviruses (Yu *et al.*, 2005). There is a discernible SCP ring around hexons in HSV-1 where this structure contributes to the shape of the outermost capsid surface. Although widely conserved, the SCP is not universally essential for virion maturation and is, for example, dispensable for HSV-1 replication in cell lines (Desai *et al.*, 1998) but essential for HCMV replication (Borst *et al.*, 2001). Thus, the nucleocapsid of every human herpesvirus consists entirely of herpesvirus-conserved proteins, but the functional requirements for capsid maturation vary to some extent.

A large number of tegument proteins are located between the capsid and envelope in herpesviruses. Tegument proteins carry out a remarkably diverse range of activities during infection, although the most well-characterized functions and the most abundant tegument proteins in any herpesvirus are often not in the core set. Eleven proteins are conserved (see Chapter 2). Many are essential for replication in those viruses that have been subjected to systematic study, HSV-1 (Roizman and Knipe, 2001), PRV (Mettenleiter, 2004) and HCMV (Dunn *et al.*, 2003; Yu *et al.*, 2003). In HSV-1, five of the conserved tegument proteins are essential for replication (HSV-1 *UL7*, *UL17*, *UL25*, *UL36*, and *UL37* gene products), including a largest tegument protein (LTP, HSV-1 *UL36* gene product) and the protein that binds to the largest tegument protein (LTPbp) encoded by the adjacent (*UL37*) gene. These play roles in entry as well as in egress. The viral serine–threonine protein kinase (VPK, HSV-1 *UL13* gene product), and five additional tegument proteins (homologues of HSV-1 *UL11*, *UL14*, *UL16*, *UL21*, and *UL51* gene products) exhibit compromised growth, sometimes in specific cell types or under certain growth conditions. In contrast, HCMV requires an overlapping, but distinct set of seven homologs to replicate, without an absolute need for some that are essential in HSV-1. In HCMV, the LTP (UL48), UL77 and UL93 (homologs of HSV-1 *UL36*, *UL25*, and *UL17*, respectively) as well as UL99, UL95, UL94, and UL71 (homologs of HSV-1 *UL11*, *UL14*, *UL16*, and *UL51*, respectively) are required for replication when the entire ORF is eliminated (Dunn *et al.*, 2003). Mutants in the LTPbp (UL47) and UL103 (homologs of HSV-1 *UL37*, and *UL7*, respectively) as well as mutants in VPK and UL88 genes exhibit a reduced level of growth. While it is possible that some of these differences stem from experimental variability or the choice of different host cells in which to study mutants, evolutionary differences are likely to dictate the extent to which each virus relies on overlapping functions as well as the extent to which functional redundancy occurs. HSV-1 UL11 and HCMV UL99 proteins are known as small myristolated tegument proteins, localize to cytoplasm of infected cells, and are involved in the latter stages of virion egress. The HSV-1, *UL11* protein interacts with two other conserved tegument proteins, *UL14* and *UL16* as well as with the envelope glycoprotein, gM, which is part of the gM/gN glycoprotein complex. Although ultrastructural analysis does not provide much information on tegument organization, an association between the LTP:LTPbp complex with the SCP ring or with triplexes in the region of the capsid vertices has been suggested with HSV-1. Investigation of tegument protein activities and capsid : tegument interactions remain important areas for experimental investigation.

One additional protein is conserved amongst herpesviruses, represented by the *UL24* gene of HSV-1 about which little is known. The HCMV homologue (UL76), a minor tegument constitutent that is essential for replication (Dunn *et al.*, 2003), localizes in a pattern that suggests it may be involved in regulating events immediately following infection or during maturation (Wang *et al.*, 2004).

One enzyme contained in this set of conserved proteins, VPK apparently acts in tandem with host cell cycle kinases to regulate a variety of replication events. VPK ensures efficient phosphorylation of other viral proteins, some of which have been reported to increase the efficiency of the host translation machinery as well as other events in gene expression and DNA replication extending from early times during infection to egress. This enzyme is dispensable for replication in alphaherpesviruses as well as in rapidly dividing host cells infected with HCMV where its role is presumably redundant with host protein kinases, possibly including Cdk2. A requirement for VPK is most readily demonstrated in primary, non-dividing host cells that have lower levels of host cell kinases. VPK is also critical for efficient replication in host animals. Interestingly, the homologue in HCMV (UL97) as well as in HHV-6B is a nucleoside kinase required for phosphorylation of the antiviral drug ganciclovir, in addition to being a protein kinase. The UL97-encoded VPK is also the primary target of a candidate antiviral compound, maribavir, which is specifically active against HCMV and EBV, but not other herpesviruses.

Human herpesvirus envelopes are estimated to carry between 12 (HSV-1) and 20 (HCMV) viral integral membrane proteins. Many envelope proteins are specific to each herpesvirus type. Five, gB, gH, gL, gM, and gN, are conserved broadly amongst herpesviruses. Sequence conservation resulted in the adaptation of a common nomenclature for structural glycoproteins, using names originally applied to HSV-1 envelope constituents. Although these names do not imply function, they are now widely used by investigators in the field. One gene product that is an O-glycosylated glycoprotein in some herpesviruses, gN, does not undergo glycosylation in some alphaherpesviruses, such as HSV-1 and VZV. Furthermore, the specific interaction between gH and gL drives stable expression of these proteins, suggesting that they are molecular chaperones as well as functional partners. A similar relationship also appears to occur with gM and gN. Although gB does not form a complex with other viral proteins, complexes of gH:gL and gM:gN form in cells and associate with cellular membranes to be incorporated into the viral envelope of progeny virions during egress. In betaherpesviruses and gammaherpesviruses, gH:gL complexes may be further modified by additional viral glycoproteins that influence cell tropism.

Entry into host cells

Attachment and entry, which typically occurs by fusion with the plasma membrane is followed by translocation of the nucleocapsid through the cytoplasm and delivery of viral genome to the cell nucleus (Fig. 4.1). This process involves a series of distinct steps that have each received varying amounts of attention in different human herpesviruses: (i) binding to specific cell surface receptors, (ii) fusion of envelope with the cellular membrane to release nucleocapsids into the cytoplasm, (iii) nucleocapsid association with cytoskeletal elements and translocation towards the nucleus, (iv) nucleocapsid interaction with nuclear pores and (v) release of the viral genome into the nucleus (see Fig. 4.1). These steps are controlled by unique as well as herpesvirus-common functions. The first step in this process involves multiple cell surface components interacting with viral envelope glycoproteins in a stepwise process that leads to membrane fusion and delivery of the nucleocapsid to the cytoplasm of host cells (Spear, 2004; Spear and Longnecker, 2003) (see specific chapters on individual viruses). Attachment to cells has been studied in most human herpesviruses and usually requires both unique and conserved, sometimes functionally redundant, viral envelope glycoproteins. With the apparent exception of EBV, cell surface proteoglycans such as heparan sulfate play a role for initial contact with cells. Heparan sulfate-dependent entry has been demonstrated in alpha- (HSV-1, VZV), beta- (HCMV, HHV-6A, HHV-6B, HHV-7) and gammaherpesvirus (KSHV/HHV-8) subfamily members (Spear, 2004; Spear and Longnecker, 2003). As a result, many herpesviruses exhibit a broad cell tropism for attachment and entry steps. In these eight human herpesviruses, gB and typically other unique viral envelope proteins exhibit heparan sulfate binding activity and direct the first attachment step in entry. Binding appears to be part of the essential role of gB in the viruses where the process has been dissected. In EBV, gB lacks the domain that controls interaction with the glycosaminoglycan and this step does not seem to be required for entry. In addition to the initial binding step, viruses where entry has been studied in detail engage additional receptors using herpesvirus-conserved as well as unique viral envelope proteins. Attachment steps may rely on unique viral envelope proteins such as EBV gp350/220, which interacts with host CD21 and EBV gH:gL:gp42 complex which interacts with MHC class II protein, KSHV K8.1A which interacts with proteoglycan, or HSV-1 gD, which interacts with nectins as well as a TNF receptor family member. In some herpesviruses conserved envelope glycoproteins are responsible for subsequent steps, such as the role of KSHV gB in binding to integin $\alpha 3\beta 1$ or the role of HCMV gB in binding to the EGF receptor, although these may be more important in the fusion step (Spear and Longnecker, 2003). Thus, except for initial contact with proteoglycan, herpesvirus attachment mechanisms appear unique to each type of virus.

In most cells that have been studied, binding through specific cellular receptors leads to fusion of the viral envelope and plasma membrane, releasing the viral nucleocapsid into the cytoplasm. Fusion typically occurs at the plasma membrane and is under the control of the herpesvirus-conserved envelope glycoproteins, gB and gH:gL which are essential for this step in all studied herpesviruses. In HCMV gH:gL may associate with additional unique proteins that provide receptor specificity, either gO encoded by UL74 or a complex of glycoproteins encoded by UL128, UL130. In HHV-6, either gO encoded by V47 or gQ encoded by U100 provide specificity. There are parallels in human beta- and gammaherpesviruses. In EBV, the presence of gp42-containing complexes reduces epithelial cell tropism, whereas the presence of gp42-free complexes reduces tropism for B lymphocytes (Borza and Hutt-Fletcher, 2002). gM:gN complex is required for entry in some settings, a feature that suggests this complex may also contribute to host cell specificity. Although entry by fusion at the plasma membrane is the most common entry

mechanism, entry via endocytosis has been characterized in some systems (Fig. 4.1). EBV entry into epithelial cells occurs by fusion directly at the cell surface whereas entry into B lymphocytes involves endocytosis (see Chapter 24). The viral functional requirements for different entry processes are still incompletely understood. Thus, both major modes of virus entry may be employed by herpesviruses depending on the setting. Signaling that results from gB or gH:gL binding to cellular receptors has also been implicated as a step in replication but no common themes have emerged from such studies. Essential core glycoproteins seem to play key roles for entry, rather than later in the replication cycle or during egress. All core glycoproteins are incorporated into infected cell membranes as well as into the virion envelope. The evolutionarily conserved manner in which gB and gH:gL control membrane fusion between the viral envelope and plasma membrane has been studied most extensively in the alphaherpesviruses where mutations in each of these gives rise to syncytial viral strains that have provided initial clues to gene products controlling this step.

Herpesviruses exploit normal cytoplasmic transport systems that control cell shape and vesicular traffic, making use of tubulin-containing microtubules and actin-containing microfilaments (Dohner and Sodeik, 2004) to control nucleocapsid transit through the cytoplasm. Like many viruses that traverse the cytoplasm, herpesviruses rely on microtubules to gain access to the nucleus and nuclear pores where uncoating is completed and the viral genome is released into the nucleoplasm. This process was suspected long ago, initially in studies with adenoviruses and herpesviruses (Dales, 1973) and has been the focus of growing attention. Microtubule-destabilizing drugs such as nocadazole and colchacine block transport and entry (Mabit *et al.*, 2002). Net transport proceeds towards microtubule minus ends that terminate at the microtubule organizing center, which is located adjacent to the nucleus. The bidirectional nature of microtubule-directed transport (Welte, 2004) allows these filaments to act as the major highways of virus particle translocation during entry as well as egress (Fig. 4.1). Microfilaments do not play as direct a role during entry; however, evidence suggests depolymerization of the actin-containing cortex may be a requisite event during entry (Jones *et al.*, 1986). Intracellular transport mechanisms that have been intensively studied in neurotropic alphaherpesviruses, predominantly HSV-1, HSV-2 and PRV, because of the requirement to translocate across long expanses of cytoplasm. Herpesvirus-conserved capsid proteins appear to rely on common cellular pathways for movement in neurons as well as all other cell types. Nucleocapsid movement occurs in both directions on microtubules (Smith and Enquist, 2002) and is likely to be regulated in ways similar to vesicle transport (Welte, 2004). Minus-end-directed transport during entry depends on the dynein:dynactin motor complex (Dohner *et al.*, 2002), which is the same motor used for directional vesicle transport. Although still controversial, contact between the SCP of HSV-1 and the cellular constituent of the dynein complex, RP3 (and possibly other proteins) has been implicated in transport (Douglas *et al.*, 2004), suggesting that this herpesvirus-conserved protein may play a similar role in other viruses. Thus, a common bridge may be built between the nucleocapsid and the microtubule to allow an appropriate direction of movement to initiate infection. Thereafter, capsid and tegument proteins act in concert to release viral DNA into the nucleus at nuclear pores although the exact process that occurs once the nucleocapsid reaches the nucleus remains largely unexplored. A conditional, temperature sensitive HSV-1 mutant together with biochemical studies have long implicated the herpesvirus-common LTP in the uncoating process (Chapter 7). The LTP of HSV-1 is needed for uncoating and release of viral DNA at nuclear pores. Studies of other tegument proteins have employed null mutants propagated on cells that complement function in egress, and result in virions that contain the protein. This approach generally masks any role tegument proteins play during entry, leaving this an important area for future exploration.

Regulation of gene expression and replication

Most regulatory proteins encoded by herpesviruses are unique. Only one core protein is purely regulatory, acting as a multifunctional regulator of expression (MRE). MRE has been most extensively studied in HSV-1 (see Chapter 9) where it is the product of *UL54* called ICP27, and EBV (Hiriart *et al.*, 2003) where it is the product of BMLF1 called EB2. MRE binds RNA and localizes to sites of transcription in the nucleus and interacts with components of the RNA polymerase II transcription machinery, the spliceosome complex and pre-mRNA export machinery. MRE stimulates late gene transcription (Jean *et al.*, 2001) and dictates the location of viral transcripts in infected cells (Pearson *et al.*, 2004). MRE is best known for impeding cellular mRNA splicing to allow the mostly intronless viral transcripts to be preferentially exported from the nucleus (Sandri-Goldin, 2001). During the early phase of infection, MRE causes splicing to stall by recruiting host cell kinases to the nucleus to inactivate splicing factors through phosphorylation. In alphaherpesvirus and gammaherpesvirus systems, MRE also recruits an export

adaptor protein (Aly/REF) to sites of viral transcription to facilitate export. This inhibition is relieved during the late phase of infection when splicing and export of host and viral transcripts resumes. Based on studies in HCMV (Lischka et al., 2006; Toth et al., 2006), the function of betaherpesvirus MRE interacts with an RNA helicase, UAP56 to promote cytoplasmic accumulation of unspliced mRNA, and this process is independent of an RNA-binding motif. The MRE may also influence shut-off of the host cell and transcriptional events through a mechanism(s) that remains to be elucidated. Although the level of sequence conservation among MRE homologs is quite limited, others appear to carry out regulatory activities and are sometimes incorporated into the virion tegument, such as in HCMV (Mocarski and Courcelle, 2001).

Viral DNA synthesis and nucleotide metabolism

All herpesviruses encode a core set of six DNA synthesis enzymes that direct the synthesis of viral DNA during productive (lytic) infection. Herpesviruses initiate lytic DNA replication at defined sites on the viral genome that are readily assayed as virus-infection-dependent autonomously replicating sequences. Some herpesviruses have a single origin of DNA replication (ori_{Lyt}), such as in the betaherpesviruses (HCMV, HHV-6A, HHV-6B, HHV-7). Others have either two (VZV, EBV, KSHV) or three (HSV-1, HSV-2) ori_{Lyt} sites that retain sequence homology, although the reason for multiple origins in the biology of viruses that carry them remains a mystery. The relative position of one copy of ori_{Lyt} adjacent to the single stranded DNA binding protein (SSB) gene is conserved in many alpha-, beta- and gammaherpesviruses, even though the primary DNA sequence of ori_{Lyt} is not conserved in all of these settings. All herpesviruses appear to rely on virus-encoded DNA binding proteins to control initiation at ori_{Lyt}. In alphaherpesviruses and the reseolavirus subgroup of betaherpesviruses, a dedicated *ori* binding protein (OBP, HSV-1 *UL9* gene product) is essential for replication. Gammaherpesviruses and cytomegaloviruses rely on DNA binding regulatory proteins that control gene expression and also function during initiation of replication.

In general, DNA replication of herpesviruses, as in other DNA viruses, starts near nuclear structures, called nuclear domain 10, which become disrupted in the course of viral infection. This process overtakes the nucleus and leads to the formation of large, distinct replication compartments where viral replication proteins and DNA accumulate (Wilkinson and Weller, 2003). Viral DNA levels can equal cellular DNA content at late times of infection. Herpesvirus DNA replication proceeds through either of two potential mechanisms that have continued to be the focus of experimental investigation. Initial models of herpesvirus DNA replication have been analogous to bacteriophage lambda (Kornberg and Baker, 1992), starting with genome circularization and theta form replication for which evidence is scant and proceeding to a rolling circle form which has been experimentally well documented (Boehmer and Lehman, 1997; Boehmer and Nimonkar, 2003; Lehman and Boehmer, 1999). This model is based on the existence of ori_{Lyt} sites and site-specific DNA binding proteins and the expectation that the viral genome circularizes upon entry into cells. More recently, a recombination-dependent branching mechanism has been proposed (Wilkinson and Weller, 2003) and supported by the failure to detect circular intermediates during infection as well as by the behavior of HSV-1 mutants that exhibit increased accumulation of circular genomes early after infection (Jackson and DeLuca, 2003). This model has been reinforced by the observation that circular forms of HSV-1 DNA do not correlate with productive replication, but rather with latency. A mechanism analogous to that in the T even bacteriophages (Kornberg and Baker, 1992) has been suggested (Wilkinson and Weller, 2003). Either mechanism of synthesis results in the production of multi-genomic length concatemers that are the substrate for progeny genome packaging using conserved viral gene products. Distinct DNA replication origins separate from ori_{Lyt} sites that are responsible for the synthesis of viral DNA during latent infection have been identified in gammaherpesviruses but not in other subfamilies.

There are two structural categories of ori_{Lyt}. In alphaherpesviruses and non-CMV betaherpesviruses that rely on OBP, the initiation of DNA replication involves a targeted unwinding to enable the assembly of a replication fork complex. This process is best understood in HSV-1. An OBP complex with the viral single stranded DNA binding protein (SSB; HSV-1 *UL29* gene product also called ICP8) unwinds DNA and binds specific sequence motifs (called Box I and Box II in HSV-1) that are symmetrically arranged within ori_{Lyt} (Macao *et al.*, 2004). This allows a more dramatic unwinding at an AT-rich region that is located between Box I and Box II followed by replication fork machinery initiating uni- or bidirectional replication (Boehmer and Lehman, 1997). Beta- and gammaherpesviruses rely on DNA-binding transactivators that are not conserved between subfamilies but act in an analogous fashion to increase transcription across the ori_{Lyt} region which opens the DNA and allows interaction with replication machinery (Xu *et al.*, 2004). Studies in HCMV, EBV and KSHV have all provided evidence for a transcriptional activator-dependent initiation that appears to be distinct

from OBP-dependent initiation in alphaherpesviruses and the betaherpesviruses like HHV-6. The betaherpesvirus HCMV encodes a virion-associated transcript that associates with ori_{Lyt} to form a three-stranded structure whose precise role in DNA synthesis is still under investigation (Prichard *et al.*, 1998). The replication fork machinery includes a highly conserved set of six herpesvirus gene products: SSB, a viral catalytic subunit of DNA polymerase (POL; HSV-1 *UL30* gene product) and associated polymerase processivity subunit (PPS; HSV-1 *UL42* gene product) and a heterotrimeric helicase-primase (HP) consisting of an ATPase subunit (HP1; HSV-1 *UL5* gene product), a primase subunit (HP2; HSV-1 *UL52* gene product) and an accessory subunit (HP3; HSV-1 *UL8* gene product). These proteins direct continuous, leading strand viral DNA replication in a rolling circle mode when used in cell-free assays (Boehmer and Lehman, 1997; Boehmer and Nimonkar, 2003; Lehman and Boehmer, 1999). Following OBP binding to specific sites in ori_{Lyt}, an interaction with single stranded DNA binding protein (SSB) leads to localized unwinding and access of replication fork proteins. Specific interaction of the HP complex with the OBP and synthesis of RNA primers may be an intermediary step leading to DNA replication mediated by the POL–PPS complex. Recombination-directed initiation may be the consequence of SSB and HP activities (Boehmer and Nimonkar, 2003) and may underlie continued viral DNA synthesis (Wilkinson and Weller, 2003; Wilkinson and Weller, 2004). Thus, these six functions provide the core DNA synthesis machinery and mediate homologous recombination during viral replication. Cellular enzymes such as ligase and topoisomerases are highly likely to be required for replication; however, a complete understanding of the steps of herpesvirus DNA replication awaits the development of defined cell-free assay conditions.

Although a high level of recombination has long been associated with herpesvirus replication (Wilkinson and Weller, 2003) and with the isolated biochemical properties of replication proteins (Boehmer and Nimonkar, 2003), only recently has this process received some support as a component of DNA replication (Jackson and DeLuca, 2003). Under conditions where replication is blocked, the HSV-1 genome circularizes more efficiently, suggesting an association of circularization with latency rather than productive replication. Though provocative, this work provides little insight into the steps required for herpesvirus replication. The circumstantial evidence that recombination plays some role either early or late in replication remains strong. In addition to its role in coating single stranded DNA at the replication fork, SSB of HSV-1 appears to direct recombination in a manner similar to *E. coli* RecA (Kornberg and Baker, 1992). Homologous recombination requires SSB as well as another herpesvirus-conserved gene product, alkaline deoxyribonuclease (NUC, HSV-1 *UL12* gene product) (Wilkinson and Weller, 2003; Wilkinson and Weller, 2004). Interestingly, this DNase is absolutely required for HCMV replication (Dunn *et al.*, 2003). In addition to the contribution of sequence specific recombination to DNA replication, circularization of the genome may itself be dependent on recombination and number of herpesviruses that undergo genome isomerization via a recombination event mediated by the *a* sequence which is located at genomic ends and at an internal junction (Mocarski and Roizman, 1982).

Two different nucleotide metabolism enzymes are also broadly conserved, deoxyuridine triphosphatase (DUT; HSV-1 *UL50* gene product) and uracil-DNA glycosidase (UNG; HSV-1 *UL2* gene product). These seem to play accessory roles in replication that are redundant with cellular enzymes. The DUT eliminates pools of dUTP, preventing the incorporation of deoxyuridine into viral DNA and produces dUMP which can be converted to TTP through cellular pathways. The DUT appears to be inactive as an enzyme in betaherpesviruses. UNG cleaves deaminated cytosines (uracil) from the sugar backbone of DNA, leading to base excision and the activation of cellular DNA repair synthesis. In cytomegalovirus, where UNG is required for replication in quiescent cells, the process of uracil incorporation and excision has been proposed to introduce strand breaks that facilitate DNA replication (Courcelle *et al.*, 2001). Functions that are important for replication in nondividing cells where cellular nucleotide metabolism enzymes would be low or absent appear to be critical for replication in the host where differentiated cells lack cellular DNA metabolism enzymes.

Finally, the large subunit of ribonucleotide reductase (RR1, HSV-1 *UL39* gene product) is conserved in all herpesviruses; however, RR1 only forms an active enzyme with a small subunit (RR2, HSV-1 *UL40* gene product) in alphaherpesviruses and gammaherpesviruses. Somewhat surprisingly, betaherpesviruses retain an RR1 that lacks enzymatic activity and do not carry a homolog of RR2 at all. RR1 may have role in cell death suppression in the betaherpesviruses as well as in some alphaherpesviruses.

Capsid assembly and DNA encapsidation

The basic features of herpesvirus capsid maturation common to all herpesviruses have been established through a combination of work with HSV-1 infected cells, recombinant baculovirus-infected cells (Thomsen *et al.*, 1994)

and, importantly, cell-free systems (Newcomb *et al.*, 1996). Assembly employs the herpesvirus-conserved components of the capsid shell (MCP, SCP, TRI1 and TRI2) working in conjunction with a precursor of the assembly protein (pAP, HSV-1 *UL26.5* gene product). Assembly of HSV-1 capsids can proceed without SCP, but at a lower efficiency. A protease (PR, also called assemblin) is required to mature the capsid. This protein is made as a precursor consisting of PR as its amino terminus fused to a longer polypeptide that contains the pAP sequence as its carboxyl end (together called prePR, e.g., HSV-1 UL26 gene product). PR is a serine protease that processes both pAP and prePR. The protease domain self-cleaves in prePR to release PR as well as a variant of pAP, and also processes the carboxyl terminus of PR, pAP, and all variants of pAP (Gibson, 1996). This processing leads to the production of multiple forms of pAP, all colinear at the carboxyl terminus. The presence of pAP is sufficient for capsid assembly, but the presence of prePR, its self-cleavage to PR and variant pAP, and its cleavage of pAP to AP are all necessary for DNA encapsidation to proceed following capsid assembly. In addition, PORT is completely dispensable for the formation of normal appearing capsids, however, this protein is absolutely required for encapsidation of viral DNA. PORT associates with pAP in order to be incorporated into capsids (Newcomb *et al.*, 2003; Singer *et al.*, 2005). During infection, pNP must be cleaved into PR and variant AP for encapsidation to follow capsid formation but both of these proteins, as well as AP, are absent from capsids once encapsidation has occurred. Phosphorylation by VPK has been implicated in the encapsidation step (Wolf *et al.*, 2001).

Once herpesvirus DNA has replicated, encapsidation is controlled by a conserved *cis*-acting element (cleavage/packaging or *pac* site) and a series of seven herpesvirus conserved *trans*-acting functions (Yu and Weller, 1998). Encapsidation has been most extensively studied in alphaherpesviruses but these studies have implications for all herpesviruses. Although initially assigned roles in viral DNA packaging, two of the conserved proteins play roles in transporting preformed capsids to DNA replication compartments, the sites of viral DNA synthesis where packaging also occurs. These two proteins, capsid transport tegument protein (CTTP, HSV-1 *UL17* gene product) and capsid transport nuclear protein (CTNP, HSV-1 *UL32* gene product) are necessary for packaging to proceed. Little is known about the way that these proteins work, except that CTTP is a virion tegument protein and may bind to the capsid (Thurlow *et al.*, 2005). CTNP is a non-structural protein and remains in the nucleus.

Packaging of progeny viral genomes follows a modified head full packaging process reminiscent of bacteriophage λ (Campbell, 1994). Capsid localization, packaging and cleavage of viral DNA are regulated by the conserved VPK as well as by cellular kinases. VPK and cellular kinases may be redundant. The packaging machinery includes a heterodimeric terminase (TER) consisting of an ATPase subunit (TER1; HSV-1 *UL15* gene product) encoded by a conserved spliced gene and a DNA recognition subunit (TER2; HSV-1 *UL28* gene product). This machinery associates with the vertex of the specialized portal penton for the introduction of a free end of a viral DNA concatemer. The PORT protein interacts with TER and brings the packaging machinery and viral DNA to the capsid vertex. This machinery controls the threading of one genome length of DNA into the capsid before scanning for a *pac* site and determining the position of DNA cleavage and therefore the genomic ends. Thus, packaging and cleavage reactions are triggered by *pac* elements located near genomic termini, typically within terminal repeats (*a* sequences). The *pac* signal is composed of at least two elements, referred to as *pac* 1 and *pac* 2, and is broadly conserved among herpesviruses such that the *pac* from HCMV can direct packaging into HSV-1 virions (Spaete and Mocarski, 1985). All studies thus far have suggested that packaging yields single base 3′ extensions at both genomic ends and that packaging proceeds directionally with regard to the viral genome orientation as originally determined for HSV-1 (Mocarski and Roizman, 1982). The conservation of signals, functions and structure strongly suggests that this process is similar across all herpesviruses.

The heterodimeric TER, which is non-structural, and the portal complex, which takes the place of one penton of the capsid, associate with two additional conserved proteins, a TER binding protein (TERbp, HSV-1 *UL33* gene product) that interacts with capsids independent of PORT and a portal capping protein (PCP; HSV-1 *UL25* gene product). PCP remains associated with the nucleocapsid after packaging and thus appears as a minor capsid protein in mature virions. These five proteins are sufficient for recognition of a *pac* site on multi-genome DNA concatamers, docking with the appropriate site on a capsid, threading viral DNA into the caspid, cleavage at a *pac* site and sealing the genome into the progeny nucleocapsid (Fig. 4.1). As such they define a set of viral functions that are likely to be required for genome packaging in all herpesviruses. Once DNA has been packaged and PCP has covered the portal, the packaging stage of replication is completed and the nucleocapsid undergoes initial envelopment at the inner nuclear membrane (Fig. 4.1).

Two additional nuclear proteins, HSV-1 *UL7* and *UL14* gene products contribute to events in capsid maturation or DNA packaging. The *UL14* gene product is a minor tegument protein that appears to act as an encapsidation chaperone protein (ECP) to bring SCP and TERbp into the nucleus (Nishiyama, 2004). The *UL7* gene product appears

to act as an encapsidation and egress protein (EEP), colocalizing with capsids in patterns that suggest a role in DNA encapsidation or egress from the nucleus.

Maturation

Following the formation of the nucleocapsid, the best evidence from several systems suggests that tegument proteins function together with non-structural proteins to control a complex two-stage envelopment and egress process that starts in the nucleus and leads to virion release by exocytosis at the plasma membrane. This two-stage envelopment process has been controversial but strong evidence has accumulated in favor of this pathway in all three herpesvirus subfamilies. The alphaherpesviruses HSV-1 and PRV have been extensively studied, along with the betaherpesviruses HCMV and MCMV (Mettenleiter, 2002; Mettenleiter, 2004). Evidence suggests that nuclear egress starts with primary envelopment at the inner nuclear membrane followed by a de-envelopment event at the outer nuclear membrane, a process that releases the nucleocapsid into the cytoplasm (Fig. 4.1). Secondary envelopment occurs in the cytoplasm at endosomal (or possibly Golgi complex) membranes with resulting vesicles carrying the fully mature virions to the cell surface using the cellular exocytic pathway. Alternatively, the older model of egress has the enveloped viral particle itself following a vesicle transport pathway without deenvelopment (Roizman and Knipe, 2001) (see Chapter 10). These steps, like the initial entry process, rely on membrane fusion events; however, envelopment and egress are relatively independent of viral envelope glycoproteins that play critical roles during entry. Non-replicating mutants in gB or the gH:gL complex mature normally but show defects in entry. Only the gM:gN complex may contribute to secondary, or final, envelopment.

Initial envelopment event occurs at the inner nuclear membrane, following and dependent upon genome packaging that produces nucleocapsids. Capsids lacking DNA do not mature efficiently and nucleocapsids must localize correctly in the nucleus to properly egress. Studies in alphaherpesviruses and betaherpesviruses have shown that two conserved proteins form a nuclear egress complex on the inner nuclear membrane to control egress from the nucleus (Mettenleiter, 2004) and disruption of the nuclear lamina. The nuclear egress membrane protein (NEMP, HSV-1 *UL34* gene product) acts as a type II membrane-spanning protein to anchor the nuclear egress lamina protein (NELP, HSV-1 *UL31* gene product), a phosphoprotein that interacts with the nuclear lamina as well as with membrane-associated NEMP. NEMP and NELP are dependent on one another for proper transport and localization to the inner nuclear membrane. Additional viral proteins or the process of viral maturation itself appear to be necessary for the formation of the nuclear egress complex. Direct binding to nucleocapsids has not been observed, however, the complex recruits viral and/or cellular protein kinases that appear to be important for phosphorylation and disruption of the nuclear lamina to allow egress. Viral mutants in NEMP or NELP are debilitated for egress, likely unable to bud through the inner nuclear membrane (primary envelopment) and so accumulate nucleocapsids inside nuclei. The nuclear egress complex may also participate in membrane fusion events at the outer nuclear membrane that are required to deposit the nucleocapsid in the cytoplasm (de-envelopment). Although NEMP, as a primary envelope protein, and NELP, as a primary tegument protein, are important components of primary virions, neither is retained in fully mature virions.

Primary envelopment delivers viral particles to the perinuclear space between the inner and outer nuclear membranes, a compartment contiguous with the endoplasmic reticulum. Over the past 10 years evidence has accumulated in several different herpesviruses that final herpesvirus envelopment occurs in the cytoplasm at late endosomal or Golgi body membranes. It is likely that primary envelopment is followed by a de-envelopment step that releases the nucleocapsid into the cytoplasm. It remains possible, but topologically unlikely that nucleocapsids move to the cytoplasm without any envelopment step through modified nuclear pores. Either way, movement of nucleocapsids to sites of final envelopment appears to be a microtubule-dependent process. Protein kinases appear to play regulatory roles in primary envelopment as well as de-envelopment occurring at the outer nuclear membrane or endoplasmic reticulum. In alphaherpesvirus, viral US3 kinase facilitates these processes by facilitating phosphorylation of NEMP or other proteins. Primary envelopment and de-envelopment may also rely on cellular kinases. In HCMV, the conserved VPK contributes to nuclear egress (Krosky et al., 2003) whereas in other betaherpesviruses such as MCMV, host protein kinase C has been implicated in nuclear egress (Muranyi *et al.*, 2002). All of this data suggests that there is redundancy and possible interplay between viral and host kinases (presumably in conjunction with host phosphatases) in establishing the appropriate phosphorylation state for egress from the nucleus.

While some virion tegument proteins are associated with nucleocapsids during nuclear egress, many are added in the cytoplasm. Major tegument proteins, though not conserved, localize to the cytoplasm in all herpesviruses that have been studied and it now appears that the bulk of tegument proteins found in mature herpesvirus virions are

added to the nucleocapsid as it traverses the cytoplasm or at sites of final envelopment. There is currently little precise understanding of how nucleocapsids engage microtubules to traverse the cytoplasm, how final envelopment at endosomal (or Golgi complex) membranes occurs or how tegument proteins might be added prior to final envelopment. There is growing suspicion that addition may be nucleated by the conserved, very large LTP:LTPbp protein complex (Mettenleiter, 2004). This and a number of additional interactions between tegument proteins (Vittone et al., 2005) may be important in function. Current evidence on alphaherpesviruses and betaherpesviruses suggests that a conserved cytoplasmic egress tegument protein (CETP, HSV-1 *UL11* gene product) plays a central role in the secondary, or final, envelopment process. In alphaherpesviruses and betaherpesviruses, CETP is a myrisoylated and pamitoylated protein that localizes to the cytoplasmic face of cellular membranes and is known to interact with another herpesvirus-conserved tegument protein, the CETP binding protein (CETPbp, HSV-1 *UL16* gene product). Together, these may form a complex involved in transport. An additional tegument protein called cytoplasmic egress facilitator 1 (CEF1, HSV-1 *UL51* gene product) is a palmitated protein that is conserved amongst herpesviruses and also associates with cytoplasmic membranes (Nishiyama, 2004). In PRV, mutants in CEF1 fail to egress properly. One final tegument protein, called cytoplasmic egress facilitator 2 (CEF2, HSV-1 *UL21* gene product) enhanced maturation and interacts with CETPbp. Limited evidence supports a role for the gM:gN complex in secondary envelopment, particularly when disrupted together with *UL11*(Kopp *et al.*, 2004; Tischer *et al.*, 2002), although this complex is dispensable for replication in a number of other alphaherpesviruses, including HSV-1. Both gM and gN are essential for HCMV replication (Dunn *et al.*, 2003) as well as for assembly in EBV (Lake and Hutt-Fletcher, 2000). gM:gN is a major EBV structural component and has recently been recognized as the major glycoprotein complex on the HCMV envelope (Varnum *et al.*, 2004).

Thus, final envelopment occurs in the cytoplasm and, as a consequence the tegument of virions includes small amounts of cellular proteins, in particular actin (Bortz *et al.*, 2003; Johannsen *et al.*, 2004; Kattenhorn *et al.*, 2004; Varnum *et al.*, 2004; Zhu *et al.*, 2005), as well as RNAs (Sciortino *et al.*, 2001; Terhune *et al.*, 2004) that may represent a quantitative sampling of the cytosol, although a role for any virion component in replication cannot be discounted as yet. Once final envelopment has occurred, exocytosis is believed to carry the mature virion inside of a vesicle to the cell surface for release. Thus, the final step in egress is fusion of an exocytic vesicle with the plasma membrane, a process that is likely to follow cellular vesicle trafficking pathways (Fig. 4.1).

This analysis of the role of herpesvirus-conserved gene products has attempted to evaluate data generated in a wide variety of systems. As a result, some data will certainly have been generalized inappropriately as further investigation reveals unique characteristics or distinguishing activities. While many common names are already in use across the different herpesviruses, the hope is that a common nomenclature for herpesvirus-common functions should emerge to facilitate comprehension of work in otherwise diverse systems. This presentation is intended to provide a starting point for evaluation of data on herpesvirus core functions no matter what virus system is being studied or discussed.

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Part II

Basic virology and viral gene effects on host cell functions: alphaherpesviruses

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5

Genetic comparison of human alphaherpesvirus genomes

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Human herpesviruses 1, *2*, and *3* (herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), and varicella-zoster virus (VZV)) have been classified as alphaherpesviruses based originally upon their biological properties, and subsequently on the sequences of their respective genomes (Minson *et al.*, 2000; Pellett and Roizman, in press). All of these viruses maintain latent infections in sensory ganglia, and can productively infect a variety of human cells, including the living cells of mucous membranes and skin. These epithelial sites also provide exit points for the virus to infect other individuals.

The structure of the genomes of the alphaherpesviruses that infect humans are quite similar at first glance (Fig. 5.1). All have two unique segments that are flanked by repeats of different lengths. The unique segments are designated short (S) and long (L) and the repeats designated as internal (IR) or terminal (TR). Members of the genus simplexvirus (HSV-1 and HSV-2) exist as four roughly equimolar isomers, each isomer differing in the relative orientations of the long and short components. The orientation of one of the HSV isomers has been designated prototypical, and could therefore be designated TRL-U_L-IRL-IRS-U_S-TRS. VZV also produces 4 genomic isomers, but those in which the long component is inverted are significantly reduced in frequency, to about 5% of total genomes. It is tempting to speculate that this is a consequence of the shorter repeats flanking the VZV long component (88.5 bp) as compared to the repeats flanking U_S in human alphaherpesviruses (6000–7400 bp) and U_L in HSV-1 and HSV-2 (around 9,000 bp). The shorter repeat region would be expected to reduce the frequency of homologous recombination events leading to less frequent inversion of the long component in VZV genomes.

The 129 kbp VZV genome is the smallest genome of the human herpesviruses, whereas the HSV-1 and HSV-2 genomes are over 152 kbp in length. The G + C content of the human simplexviruses is around 68% whereas the VZV genome is only 46% G + C. Thus, considerable numbers of mutations have occurred since the two lineages arose from a common progenitor. All three viruses encode well-conserved sequences that guide lytic replication (oriLyt) and genome cleavage and packaging (Frenkel & Roffman 1996). Copies of oriLyt are present in IRS and TRS of all three viruses, and near the center of L for HSV-1 and HSV-2.

Several features are apparent in the global sequence comparisons shown in Fig. 5.2. HSV-1 and HSV-2 are much more closely related to each other than is either to VZV. HSV-1 and HSV-2 genomes are most similar in their U_L components, and least similar in the inverted repeats that bound U_L (TRL and IRL). The VZV sequence is most closely related to HSV in their U_L components. These observations can be extended by measuring the level of sequence identity along the dot plot diagonal, as shown in the nucleotide sequence similarity plot for HSV-1 vs. HSV-2 (Fig. 5.3). As can be seen, the similarity is highest across U_L, with most of the peaks corresponding to protein coding regions, and the valleys to intergenic regions, the exceptions being U_L 42 through U_L 44, and U_L 49. The L component repeats are much less well conserved, with the peak of similarity being in the region encoding ICP0 (RL2). The most highly conserved region in the S component repeats is the ICP4 gene (RS1). Overall, U_S is less well conserved than U_L. This region includes the U_S 4 (gG) gene, which is approximately 1500 bp longer in HSV-2 than in HSV-1 and represents the region of greatest dissimilarity between HSV-1 and HSV-2.

The gene arrangement in the long component of the most common isomer of the VZV genome is inverted relative to the U_L of the prototypical isomer of HSV (Fig. 5.1). With this inversion in mind, the genes in the HSV-1 and HSV-2 U_L segments are mostly collinear with VZV U_L (Fig. 5.2), and all but a few U_L genes are conserved across all human alphaherpesviruses. This consideration also underscores the significance of the overall similarity of the genes,

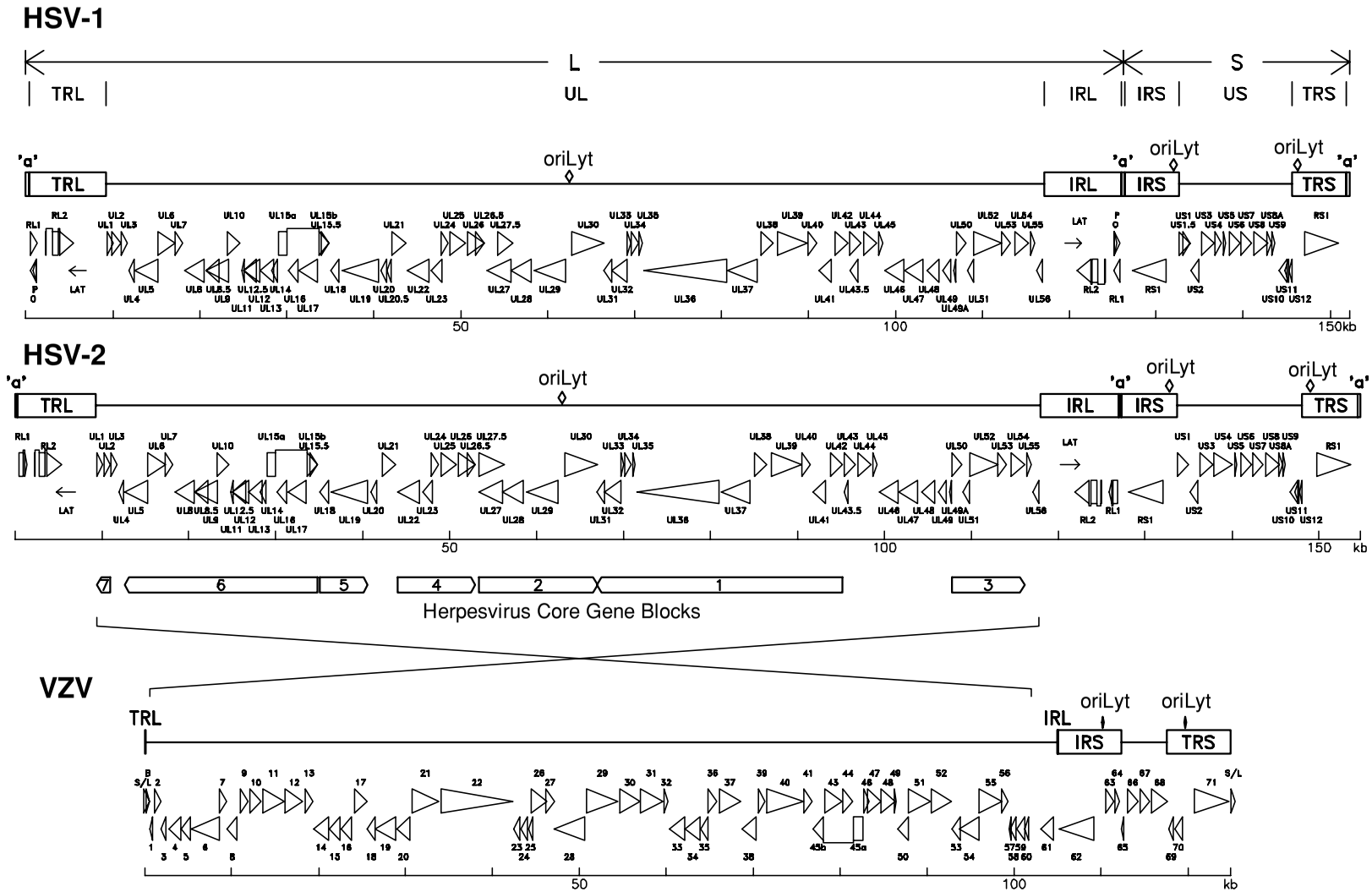

Fig. 5.1. Genomic and genetic architectures of the human alphaherpesviruses. Note that the VZV L component is inverted relative to the HSV prototypic arrangement. The representations are based primarily on annotations in Genbank Accession numbers X14112 (HSV-1 strain 17), Z86099 (HSV-2 strain HG52), and X04370 (VZV strain Dumas), and information in references cited in the text. L, long component; S, short component; TRL, long component terminal repeat; IRL, long component internal repeat; IRS, short component internal repeat; TRS, short component terminal repeat.

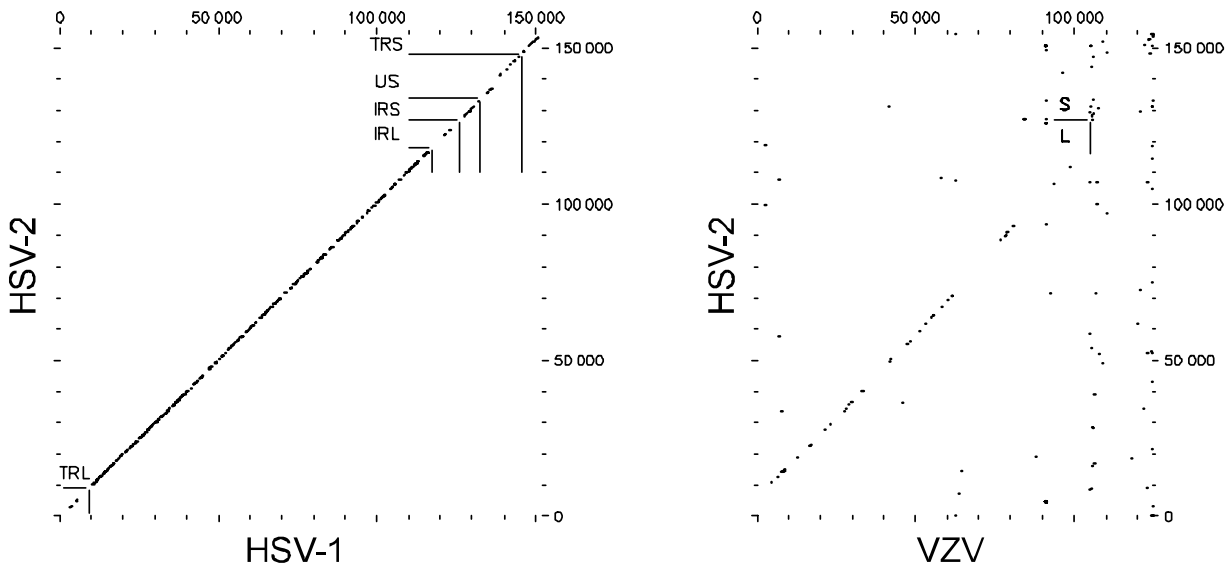

Fig. 5.2. Global genome sequence similarity comparisons among the human alphaherpesviruses. Dot similarity plots were constructed from comparisons of complete genome sequences. For HSV-1 vs. HSV-2, the window was 50 and the stringency was 45 identical residues. For HSV-2 vs. VZV, the window was 50 and the stringency was 25 identical residues. This reduced stringency was needed, because there were no dots when the HSV-2 and VZV were compared at the same stringency used for the HSV-1/HSV-2 comparison. Because the prototypic genomes of HSV-2 and VZV have relatively inverted L components, the HSV-2/VZV comparison was done with a VZV sequence in which the L component was inverted. Sequence sources and abbreviations are the same as for Fig. 5.1.

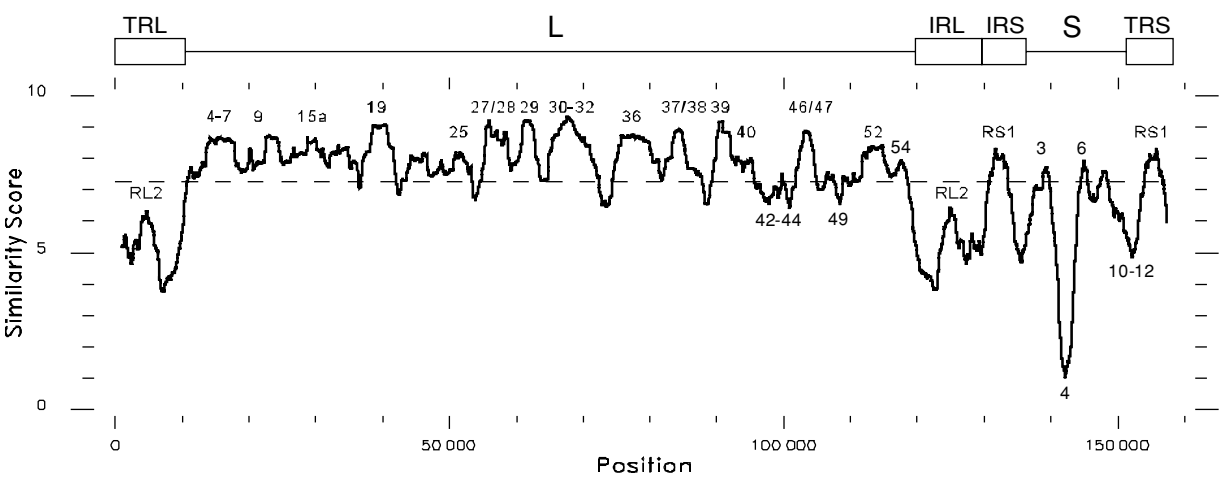

Fig. 5.3. Nucleotide sequence similarity between HSV-1 and HSV-2. HSV-1 and HSV-2 nucleotide sequences were aligned in approximately 20 kb segments, the aligned segments were joined to generate genome-length alignments, and then the similarity along the aligned genomes was plotted as a running percentage of identity in windows of 2000 residues. x-axis positions do not correspond precisely to genomic coordinates because of spaces inserted during the alignment. Boundaries of major architectural features are indicated, as are the locations of various genes along the plot. Sequence sources and abbreviations are the same as for Fig. 5.1.

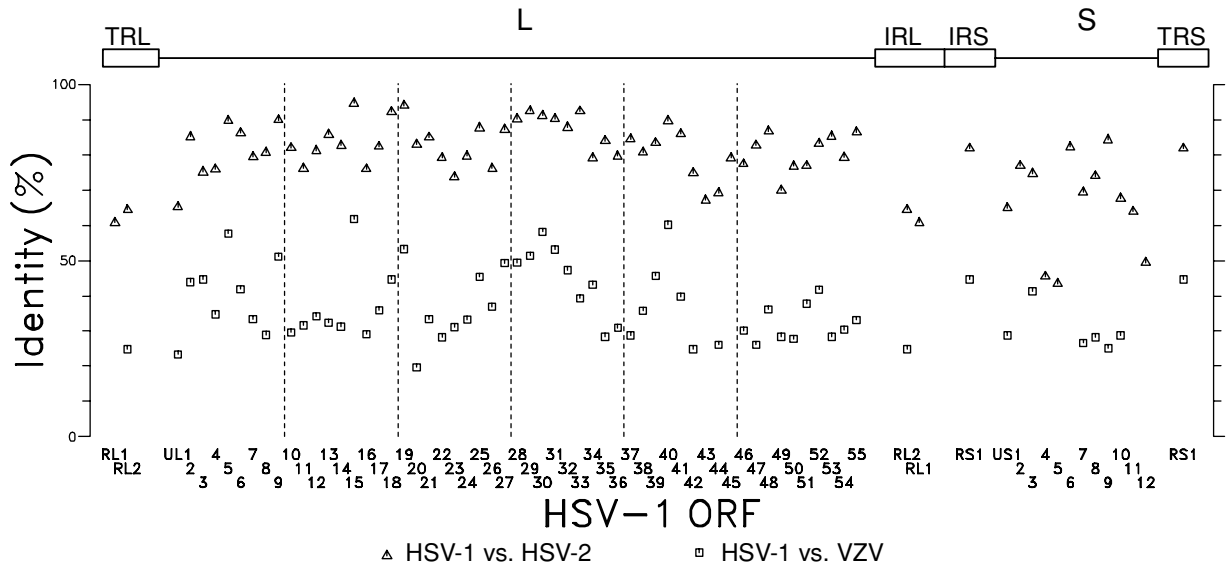

Fig. 5.4. Amino acid sequence identities between proteins encoded by the human alphaherpesviruses. Homologous protein sequences were aligned, and the percentage of identical residues determined in comparisons between HSV-1 and HSV-2 (triangles), and between HSV-1 and VZV (squares). Identity scores were plotted as a function of their location in the HSV-1 genome, relative to the major genomic architectural features. As detailed in the text, some HSV genes do not have identified homologues in VZV, and vice versa, and some genes are encoded within and in frame with others (and are thus not represented individually in this figure). Sequence sources and abbreviations are the same as for Fig. 5.1.

suggesting that they are considerably constrained by their respective functions. The most highly conserved genes between VZV and HSV-1 (HSV U_L 5, U_L 15, U_L 30, and U_L 40) are among the most highly conserved between HSV-1 and HSV-2 (Fig. 5.4). Their encoded proteins are enzymes involved in DNA replication and metabolism. This suggests that there is both little external pressure for these genes to change and little tolerance for accepting the products of random mutation. The least conserved genes between HSV-1 and HSV-2 are encoded in the S component and TRL/IRL; as detailed below, many of these genes do not have homologues between HSV and VZV. These gene products are likely to be important in defining the precise biological niche occupied by each virus.

VZV genes that are absent from HSV genomes

Although homologues of most long component genes exist in HSV-1, HSV-2 and VZV, there are some notable exceptions. Six VZV U_L genes have no homologue in the HSV U_L (ORF1, ORF2, ORF13, ORF32, ORF57 and ORF S/L). All of these genes are dispensable for replication in cell culture in at least some cell types.

ORF1 encodes an integral membrane protein of approximate M_r 17 000 with a C-terminal hydrophobic domain. Its function is not known, but it is completely dispensable for growth of the virus in MeWo cells (Cohen and Seidel, 1995). Equine herpesvirus 1 (EHV-1) and EHV-4 are both members of the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae* (Telford *et al.*, 1992, 1995). Although these viruses encode a gene (termed EHV gene 2) in a position that corresponds to VZV ORF1, it has no obvious similarity with the VZV counterpart.

VZV ORF2 also encodes a phosphorylated membrane-associated protein that is dispensable for growth in cell culture and establishment of latency in dorsal root ganglia of cotton rats (Sato *et al.*, 2002). Genes with very limited homology with VZV ORF2 are present in other Varicelloviruses including gene 3 of EHV-1 and EHV-4. The functions of these genes are not known.

VZV ORF13 encodes thymidylate synthetase. Interestingly, homologues are not present in other alphaherpesviruses, but the genomes of several gammaherpesviruses, including human herpesvirus 8, herpesvirus saimiri, herpesvirus ateles, and equine herpesvirus 2 contain functional homologues. Moreover, human cytomegalovirus upregulates cellular thymidine synthetase (Gribaudo *et al.*, 2002). Thus, incorporation into the viral genome, or other means of upregulation of thymidylate synthetase in the host cell likely confers a selective advantage to these

viruses, possibly as it promotes availability of nucleotides for viral DNA replication in quiescent cells. Within the region corresponding to VZV ORF13, HSV-1 and HSV-2 encode U_L 45, which is encoded on the opposite strand and has no homologue in VZV. The U_L 45 gene encodes a type II membrane protein that is dispensable for replication in cell culture (Cockrell and Muggeridge 1998; Visalli and Brandt 1991).

VZV ORF32 is homologous to gene 34 of EHV-1 and EHV-4, but has no counterparts in other herpesvirus genera. The encoded protein of 16,000–18,000 M_r is posttranslationally modified by the ORF47 protein kinase and is dispensable for change replication in cell culture and the establishment of latency (Reddy *et al.*, 1998; Sato *et al.*, 2003).

VZV ORF57 is located in a region of unusually high diversity among the U_L segments of alphaherpesvirus genomes. VZV ORF57 has no sequence similarity to genes in herpesviridae for which DNA sequence data are available. In HSV, the corresponding region encodes no obvious open reading frame, and in fact has served as an insertion site for the expression of exogenous genes and bacterial artificial chromosome vector sequences without obvious detriment to the virus in cultured cells or animals (Baines and Roizman, 1991; Tanaka *et al.*, 2003). On the other hand, the region between U_L 3 and U_L 4 in the Suid herpesvirus 1 (Pseudorabies virus or PRV) genome encodes gene U_L 3.5 that is necessary for viral egress (Fuchs *et al.*, 1996). Like VZV ORF57, the PRV U_L 3.5 has no obvious homologue in any other herpesvirus.

ORF S/L is also unique to VZV. The encoded protein is located in the cytoplasm of infected cells and varies in size from about 21 000–30 000 apparent M_r among different VZV strains (Kemble *et al.*, 2000). The initiation codon of ORF S/L lies at the right end of TRS with translation crossing into the L component (this requires genome circularization or concatemerization). VZV ORF B is translated from within the same ORF, but initiates within the L component, terminating at the same stop codon as ORF S/L (Mahalingam *et al.*, 2000). The functions of either protein are unknown.

L component genes unique to the simplexviruses

Unless otherwise noted, it should be assumed that the following genes are encoded by HSV-1 and HSV-2, but not VZV.

The 248 residue ORF P lies in the repeats bordering U_L (TRL and IRL). The associated transcript is antisense to the RL1 gene encoding γ_1 34.5, and ends where the full length latency associated transcript (LAT) terminates. The gene is expressed primarily under conditions where other HSV genes, including those of the immediate early or alpha class, are not expressed (Lagunoff and Roizman, 1994). Deletion of an ICP4 binding site in the ORF P promoter augments expression, suggesting that ICP4 normally acts to repress ORF P (Lagunoff *et al.*, 1996). Because HSV alpha genes are not expressed during latent infection, it has been speculated that ORF P might be expressed preferentially in latently infected neurons, although this has not been demonstrated. The predicted HSV-2 counterpart of ORF P is 130 amino acids in length and has substantial similarity over the first 49 codons, after which the similarity drops off significantly at a site corresponding to an intron in HSV-2 RL1, which is encoded on the opposite strand (Dolan *et al.*, 1998). It has not been determined if the HSV-2 counterpart to ORF P is expressed.

ORF O of HSV-1 is shorter than, and entirely contained within, ORF P. The protein is regulated in a similar fashion to ORF P, and appears to be translated from the same inititation codon, but shifts frame by an unknown mechanism after codon 35 to a different reading frame, resulting in translation of a 20 000 apparent M_r protein. The protein can interact with ICP4 and preclude the latter's binding to DNA (Randall *et al.*, 1997). Similar to ORF P, the homology to an HSV-2 counterpart is only convincing at the extreme N-terminus and it is not known if the HSV-2 protein is expressed.

The γ_1 34.5 protein is encoded by RL1 in the repeat region, with the promoter located in the a sequence, and the open reading frame in the b sequence. The protein is a major determinant of neurovirulence and has a number of interesting functions including the recruitment of phosphatase alpha to dephosphorylate elongation initiation factor alpha and thus preserve translation of viral proteins, even in the presence of PKR (He *et al.*, 1997). The HSV-2 counterpart contains an intron, but is otherwise conserved. There is no obvious homologue in VZV or in herpes B virus (Perelygina *et al.*, 2003).

The U_L 8.5 gene is present in both HSV-1 and HSV-2. The gene product, designated OBPC, represents the C terminal 438 amino acids of the U_L 9 gene that encodes the origin binding protein (OBP). OBPC can bind origin sequences and can interfere with DNA replication in vitro (Baradaran *et al.*, 1996; Baradaran *et al.*, 1994).

The U_L 12.5 open reading frame is translated in frame with U_L 12 to yield a 60 000 M_r protein. It retains some of the nuclease activity of U_L 12, but lacks a nuclear localization signal at the N-terminus, perhaps explaining why U_L 12.5 cannot complement a U_L 12 null mutant. The precise function of U_L 12.5 is not known (Martinez *et al.*, 1996; Reuven *et al.*, 2004).

The U_L 15.5 open reading frame is present in VZV, but in contrast to the case with HSV-1, it is not known if the VZV protein is expressed. The open reading frame constitutes most of exon II of U_L 15 and the protein is in frame with U_L 15. The function is not known (Baines *et al.*, 1994, 1997; Yu *et al.*, 1997).

The U_L 20.5 open reading frame is located upstream of U_L 20. The gene product is expressed in infected cells and localizes in discrete sites in the nucleus. The open reading frame is not conserved in HSV-2 or VZV and the function is not known (Ward *et al.*, 2000).

U_L 27.5 is encoded in opposite sense to U_L 27, which encodes glycoprotein B. The 43 000 apparent M_r protein identified in infected cells is much smaller than the 575 codon open reading frame would predict. An HSV-2 protein of similar size that shares epitopes with the HSV-1 gene product is derived from an open reading frame of 985 codons (Chang *et al.*, 1998). The mechanism by which these large open reading frames lead to production of smaller than expected proteins is unknown.

U_L 43.5 is a 311 aa ORF that is encoded completely within U_L 43 coding sequences but is translated in opposite sense to that of U_L 43 (Ward *et al.*, 1996). The genomic region encoding these open reading frames is dispensable for viral growth in cell culture (MacLean *et al.*, 1991). The protein localizes in assemblons, discrete sites within infected cell nuclei that contain a variety of capsid and tegument proteins. The U_L 43.5 open reading frame is not conserved in HSV-2 (Dolan *et al.*, 1998).

The U_L 56 gene is unique to the simplexviruses of humans. The gene lies in a region that is necessary for a virulent phenotype of certain HSV-1 strains, but the gene itself does not contribute substantially to this virulence, at least in mice (Nash and Spivack, 1994). The gene product is associated with virions and is not essential for growth in cultured cells (Rosen-Wolff *et al.*, 1991).

The unspliced latency associated transcript or LAT is around 7–9 kbp in length, and this is extensively spliced. The introns of approximately 1.5 and 2.0 kbp are presumably very stable and accumulate to high levels in the nuclei of latently infected sensory neurons. Both the large and small transcripts are derived from transcriptional units within IRL and TRL and are transcribed in a sense opposite to that of RL1 (encoding ICP0) with which they overlap. Transcription through the repeat regions and antisense to ICP0 is a common theme among many alphaherpesviruses including bovine herpesvirus 1 and PRV, but these transcripts are not greatly similar to HSV LAT, other than in regions that overlap conserved open reading frames (Cheung, 1991; Rock *et al.*, 1987). More extensive discussions of the latency associated transcripts are included elsewhere in this volume.

S component genes unique to the human simplexviruses

The short components of the human alphaherpesviruses are considerably less well conserved than the long components (Figs. 5.1 to 5.4). Perhaps the most striking difference is that homologues of six HSV genes, including U_S 6 encoding the essential glycoprotein D, are not present in the VZV genome (Dolan *et al.*, 1998; McGeoch *et al.*, 1988). In addition, the order of the existing homologues of U_S is considerably rearranged (Davison and McGeoch, 1986).

The U_S1 gene of 420 codon encodes ICP22 whereas the $U_S1.5$ open reading frame of 273 codons is contained within the U_S 1 gene, and is translated in the same reading frame as ICP22 but from a different initiation codon (Carter and Roizman, 1996). A homologous, but highly diverged gene to that encoded by U_S 1.5 is present in HSV-2, but this lacks an obvious start codon. The VZV counterpart is ORF 63/70 (the gene is duplicated at the ends of the short component of VZV); the region of highest similarity with HSV-1 is limited to the C-terminus which includes US1.5.

The U_S2 gene of HSV is present in many other alphaherpesviruses, but is conspicuously absent from the VZV genome. The function of the gene is not known but the gene product is found in HSV-2 virions and can associate with cytokeratin 18 in vitro and in infected cells (Goshima *et al.*, 2001). U_S2 is not essential for growth in cell culture and the HSV-2 gene is dispensable for virulence in mice inoculated by the footpad route (Jiang *et al.*, 1998; Longnecker and Roizman, 1987). The homologous gene product of pseudorabies virus (PRV) is prenylated, a modification that changes its localization from association with membranes to punctate regions in the cytoplasm (Clase *et al.*, 2003). The gene product is associated with virions and this association may be regulated by postranslation modification, inasmuch as the virion-associated gene product is not prenylated.

The U_S 4 gene encodes glycoprotein G (gG). The gene is dispensable for growth in cell culture and differs significantly in sequence such that the HSV-2 gene is approximately 1500 bp larger (Ackermann *et al.*, 1986; Dolan *et al.*, 1998; Longnecker *et al.*, 1987; McGeoch *et al.*, 1988). This difference is exploited in serologic assays to distinguish HSV-2 specific antibodies from those induced by HSV-1

(Lee *et al.*, 1985). The function of gG is not known, but speculation that it has something to do with tropism unique to HSV-2 vs. HSV-1 seems warranted.

U_S5 encodes glycoprotein J (gJ) (Ghiasi *et al.*, 1998). gJ prevents apoptosis in cells infected with HSV-1 gD null mutants, and precludes apoptosis induced by granzyme B and Fas ligation, such as would be expected upon attack of an infected cell by cytotoxic T-lymphocytes (Jerome *et al.*, 2001; Zhou *et al.*, 2000). Interestingly, HSV-2 does not preclude apoptosis induced by ultraviolet radiation or Fas antibody, whereas HSV-1 blocks apoptosis through these stimuli, suggesting a difference in function of anti-apoptotic mechanisms mediated at least partly through gJ (Jerome *et al.*, 1999).

U_S6 encodes gD that is necessary for HSV entry and has been shown to bind a variety of proteinaceous viral receptors (Spear, 2004). The fact that a gene essential for entry of HSV is absent from the VZV genome suggests that the essential steps mediated by gD must be mediated by different VZV proteins. This issue is treated extensively in chapters dealing with herpesvirus entry.

$U_S8.5$ encodes a phosphoprotein that localizes to nucleoli (Georgopoulou *et al.*, 1995). The gene is dispensable for replication in cultured cells and the function is not known. Although $U_S8.5$ is a late gene, the mRNA is packaged into virions and presumably delivered to infected cells upon entry (Sciortino *et al.*, 2002).

The 161 codon U_S11 of HSV-1 is an RNA binding protein that is unique to the simplexviruses of humans. If expressed as an alpha gene, the protein can dephosphorylate EIF2α and thereby rescue the ability of a γ_1 34.5 deletion mutant to replicate in neuronal cell lines (Mohr and Gluzman, 1996; Roller *et al.*, 1996).

U_S12 encodes ICP47, an 88 amino acid immediate early protein. ICP47 can block the transporter associated with antigen transport (TAP) and thus preclude loading of antigenic peptides onto class 1 molecules in the ER (York *et al.*, 1994). Although ICP47 may be unique to human simplexviruses, it can be viewed as functionally conserved inasmuch as interference with antigen presentation is a function common to many viral proteins, including a number from both gamma- and betaherpesviruses (Vossen *et al.*, 2002).

In considering the information outlined herein, the authors would like to include a note of caution. While comparison of the sequences of the human alphaherpesviruses with one another and with other herpesviruses leads to a series of hypotheses regarding functions of individual genes, such an analysis is at once both potentially valuable and misleading. Fortunately, such hypotheses are experimentally testable in the context of the most relevant viral genome. It is anticipated that some surprises will result from these studies. Perhaps this is best illustrated by studies of herpesvirus glycoproteins, as various sequence homologues may have markedly different functions depending on the viral system studied. At the least, the preceding analyses of similarities in the genetic content of the human alphaherpesviruses should be considered with this caveat in mind.

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6

Alphaherpes viral genes and their functions

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Introduction

In this chapter the emphasis is on viral replication and on the viral gene products that define the outcome of the interaction of the alphaherpesviruses with their host. Viral replicative and host management functions account for some of the RNAs and a large number of proteins encoded by the viruses. There are, however, numerous viral gene products whose functions have not been identified or which do not play a prominent role in viral replication in the systems in which these have been tested. The objective of the table contained in this section is to summarize the functions of all known gene products and provide at least a few references for each product. It should be noted however that: of the three human alphaherpesviruses, we know more about the functions of herpes simplex virus-1 and -2 (HSV-1 and HSV-2) genes than about those of varicella zoster virus (VZV). We have identified in this table the VZV genes that are related to HSV by amino acid sequence homology. We note that partial sequence conservation does not necessarily mean that the homologous HSV and VZV gene products perform identical functions.

The list understates both the number of the products and their functions. The problem is twofold. The HSV genome encodes a large number of open reading frames (ORFs) with 50 or more codons and not all of the ORFs have been probed for to determine whether they are expressed. In addition, standard annotations exclude ORFs that are antisense to known ORFs or that do not have TATA boxes or other motifs that indicate that they encode proteins. HSV encodes several proteins whose ORFs are antisense to each other. An additional problem is that transcripts arising late in infection frequently do not terminate at predicted termination signals. In addition, several viral RNAs either do not encode a protein or the protein is made in undetectable amounts. In several instances, ORFs contain within their domain a transcriptional unit encoding proteins identical to the C terminal domain of the protein encoded by the larger ORF. In essence, we do not know the absolute number of transcripts or proteins encoded by the viral genome.

The third limitation of the table stems from the observation that virtually all viral proteins studied in detail appear to perform multiple functions. Not all of the functions are known and, in some instances, the table lists the most prominent functions of the gene product.

We further note that (i) some alphaherpesvirus genes are found only in HSV-1 (ORF-O and ORF-P), some in HSV-1 and HSV-2 ($\gamma_1$34.5), some in HSV-1, HSV-2 and B virus (the simplexviruses), some in the simplexviruses plus pseudorabies virus, and some are in all of the above, plus VZV. This is annotated in the "Conservation" column. (ii) Some genes have positional homologues (conserved size, orientation, and conserved surrounding genes) for which no functional data are available. We included positional homologues, in cases in which good candidates are present in members of all three herpesviruses subfamilies.

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d]				Ref.
					VZV	γ-HV	HCMV	HHV-6	
$\gamma_1$34.5		This ORF encodes a 248-residue γ_1 protein consisting of two unequal domains linked by a variable number of alanine-proline-threonione repeats. The C-terminal domain is homologous to the C-terminal domain of GADD34 and functions as a phosphatase accessory factor which binds phosphatase 1α and redirects it to dephosphorylate the α subunit of the translation initiation factor 2 (eIF-2). In the absence of $\gamma_1$34.5 gene, protein kinase R is activated, eIF-2α is phosphorylated and all protein synthesis ceases. The loss of this function is responsible for the loss of the capacity of mutants to replicate in experimental animal systems (loss of neurovirulence). $\gamma_1$34.5 protein is also involved in the evasion of MHC class 2 responses and encodes other, as yet poorly defined functions. γ_1.[a]	HSV-2, Gadd34 (MyD116)						(100, 101, 230)
ORF-P		A 233-residue ORF encoded on the strand complementary to $\gamma_1$34.5. The transcription of ORF-P is blocked by ICP4 bound to a high affinity transcription initiation site of the ORF. Derepression of ORF-P results in decreased expression of the $\gamma_1$34.5 ORF. ORF-P protein binds p32 and localizes in spliceosomes. Overexpression of ORF-P results in decreased expression of viral products of spliced RNAs. Pre-α.	not in HSV-2 or B virus						(128, 129)
ORF-O		The product of ORF-O is made of 117 residues. The amino terminus is identical to that of ORF-P. The amino acid sequence beyond residue 35 is in an alternate reading frame. Expression of ORF-O is repressed by ICP4. In vitro ORF-O blocks the binding of ICP4 to its cognate high affinity DNA binding sites. Viral gene products encoded by these ORFs have not been detected in murine ganglia harboring latent virus. Pre-α.	not in HSV-2 or B virus						(198)
α0	ICP0	The 3 exons encode a 775-residue protein containing a RING finger domain. ICP0 is dispensable for viral replication in cultured cells. In transfected cells it acts as a promiscuous transactivator of genes introduced by transfection or infection although activation of resident genes has been reported. ICP0 expresses multiple functions, including those of a double ubiquitin ligase targeting promyelocytic leukemia protein to block exogenous interferon, cdc34 to block turnover of cyclin D3, CNP-C, CNP-A, and DNA dependent protein kinase. ICP0 is also associated with the restructuring of chromatin. Additional functions have been described. α	α-HV		ORF61				(99, 171, 222)
U_L1	gL	224 residue soluble glycoprotein. It interacts with and serves as the chaperone of gH. In its absence gH is not transported to plasma membrane and is not fully glycosylated. Essential glycoprotein for virion infectivity and cell-cell fusion. Locus of syncytial mutation. Stimulates neutralizing antibody. γ_1.	α-HV, β-HV, γ-HV	7	ORF60	ORF47	UL115	U82	(78, 106, 203)

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d]				Ref.
					VZV	γ-HV	HCMV	HHV-6	
U_L2		U_L2 encodes uracil-DNA glycosylase, a highly conserved enzyme associated with the base excision repair pathway. Uracil-DNA glycosylase replaces uracil in G:U base pairs resulting from deamination of cytosine residues in DNA. U_L2 is highly conserved and no other functions have been ascribed it. 334 aa. β or γ_1.	α-HV, β-HV, γ-HV.	7	ORF59	ORF46	UL114	U81	(41, 244)
U_L3		U_L3 is a 235 residue phosphoprotein forming multiple bands in denaturing polyacrylamide gels due in part to phosphorylation mediated by U_L13 protein kinase. It co-localizes with ICP22-$U_S1.5$ proteins in small dense nuclear structures. In the absence of ICP22 or $U_S1.5$ it is diffuse throughout the nucleus. The function is unknown: the gene can be deleted without impairment of viral replication in cultured cells. The protein is transcribed predominantly from the second methionine of its reading frame. γ_2	α-HV Possible positional homologue in γ-HV		ORF58	ORF45			(10, 152, 245)
U_L4		U_L4 is a 199-residue virion protein. In infected cells it localizes in small dense nuclear structures formed prior to the onset of viral DNA synthesis together with ICP22/$U_S1.5$. In the absence of other viral proteins it remains in the cytoplasm. U_L4 and is dispensable for viral replication in cultured cells. γ_2	α-HV		ORF56				(110, 113, 249)
U_L5		U_L5 is a component of the helicase – primase complex. The 882-residue protein contains sequence motifs shared by members of the superfamily of RNA and DNA helicases ranging from bacteria to mammals. Stable U_L5-U_L52, complex has DNA-dependent ATPase and GTPase, DNA primase and DNA helicase activities. A viral mutant in U_L5 exhibiting a neuron specific restriction was shown to produce lower levels of viral DNA in restricted cells. β.	α-HV, β-HV, γ-HV	6	ORF55	ORF44	UL105	U77	(28, 76)
U_L6		The 676-residue protein forms a dodecameric ring located at the vertices of the HSV capsid. The ring structure is similar to the portal for the entry of DNA in bacteriophages. U_L6 has been reported interact with U_L15 and U_L28 (the putative terminase) proteins. Resistance of a class of thiourea drugs that blocks cleavage and packaging of DNA was mapped to U_L6 ORF. Unknown.	α-HV, β-HV, γ-HV	6	ORF54	ORF43	UL104	U76	(176, 232, 242)
U_L7		The 296-residue protein is well conserved among herpesvirus families and may be a component of the tegument. Its function is not known. It is not essential for replication in cell culture. γ_1.	α-HV, β-HV, γ-HV	6	ORF53	ORF42	UL103	U75	(181)
U_L8		The 750-residue U_L8 protein is required for the transport of U_L52 and U_L5 to the nucleus. It interacts with both U_L9 and U_L30. One likely function of U_L8 protein is to facilitate the synthesis of RNA primers on the DNA template. β.	α-HV, β-HV, γ-HV	6	ORF52	ORF41	UL102	U74	(18, 40, 153, 159, 229)

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d]				Ref.
					VZV	γ-HV	HCMV	HHV-6	
U_L8.5		The 487-residue U_L8.5 protein corresponds to the C-terminal domain of U_L9 protein. U_L8.5 was reported to be synthesized both early and late in infection and to bind to the origin of DNA synthesis. In transient assays the U_L8.5 protein inhibited DNA synthesis. It may play a role in shifting the pattern of synthesis for de novo initiation at origins to a rolling circle model of viral DNA synthesis. γ_1.	α-HV						(15, 16)
U_L9		U_L9, a 851-residue protein, binds to two specific sites flanking the origin of viral DNA synthesis. ICP8 (U_L29) which binds and separates the DNA strands, enables the unwinding of the DNA at the site of the origin by U_L9 protein and allows the entry of the machinery that replicates the DNA. U_L9 is conserved in α-herpesviruses and except for HHV6 and HHV7 it is absent from the genomes of β- or γ-herpesviruses. The function of U_L9 after the initiation of viral DNA synthesis has not been demonstrated. U_L9 protein was reported to interact with two proteins. hTid-1 enhances the binding of a multimer of U_L9 to the origin of DNA synthesis. NFB42, a F box component of the SCF ubiquitin ligase, binds phosphorylated U_L9 protein and targets it for ubiquitin-proteasomal degradation. The phosphorylation and degradation of U_L9 may be a mechanism by which the virus favors a rolling circle type of DNA replication instead of de novo initiation of DNA synthesis at the origins or replication. β.	α-HV, Roseolo-viruses	6	ORF51			U73	(50, 81, 126, 132, 148, 224, 238)
U_L9.5		The transcript of U_L9.5 is coterminal with those of U_L8, U_L8.5, and U_L9. 472 aa. γ_2							(16)
U_L10	gM	Abundant virion glycoprotein of 473 residues, with apparent M_r 53–63,000. Topology predicts six to eight transmembrane segments. A deletion mutant virus has no major phenotype. In other herpesviruses, gM forms a complex with gN, or gN-gO. Based on phenotype of ΔgM-gE-gI PrV and ΔgM EBV viral mutants, gM is thought to play a role in virion maturation and exocytosis, but this function may be redundant. However, a ΔgMgE HSV mutant shows no defect. $\gamma_.$	α-HV, β-HV, γ-HV	6	ORF50	ORF39	UL100	U72	(11, 35)
U_L11		U_L11 is a 96-residue tegument protein that is both myristoylated and palmitoylated. ΔU_L11 mutants exhibit reduced levels of envelopment and egress of virus from infected cells. The protein binds the cytoplasmic face of cellular membranes and is particularly abundant in Golgi. U_L11 protein interacts with and directs U_L16 to the Golgi. The precise function of U_L11 is not known. $\gamma_.$	α-HV, β-HV, γ-HV	6	ORF49	ORF38	UL99	U71	(12, 140, 141, 143, 144)
U_L12	Alkaline nuclease	U_L12 is a 626-residue alkaline nuclease that functions as a resolvase, an enzyme required for processing of replication intermediates-structures defined by their inability to enter pulse field gels and that would interfere with the packaging of viral DNA into capsids. β.	α-HV, β-HV, γ-HV	6	ORF48	ORF37	UL98	U70	(93, 154, 241)

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d]				Ref.
					VZV	γ-HV	HCMV	HHV-6	
$U_L12.5$	No VZV	The ORF encodes a 500-residue product from a 1.9 kb RNA and corresponds to the C-terminal portion of the U_L12 alkaline nuclease. The protein does not have nuclease activity, does not complement U_L12-null mutants and does not appear to be a structural component of capsids. Its function is not known. Unknown							(154)
U_L13	Protein kinase	U_L13 encodes a 518-residue (M_r 56,000) serine/tyrosine protein kinase for viral and cellular proteins. Its function as a tegument protein is unknown. The gene is essential for viral replication in experimental animal systems but not in cells in culture. U_L13 appears to regulate multifunctional proteins. The substrate specificity of U_L13 protein kinase is similar to that of the mitotic cyclin dependent kinase cdc2. (Y. Kawaguchi, personal communication). γ_1.	α-HV, β-HV, γ-HV	6	ORF47	ORF36	UL97	U69	(25, 67, 116, 117, 196, 217)
U_L14		The ORF encodes a tegument protein of 219 residues. ΔU_L14 mutants replicate well in cell culture but are highly attenuated in the mouse. The only function attributed to U_L14 protein is enhancement of nuclear localization of U_L17 and U_L26. Co-expression of U_L14 enhances nuclear import of the U_L35 (VP26) and U_L33 proteins and increases luciferase expression suggesting that it may facilitate folding of a variety of proteins. γ_2.	α-HV, β-HV, γ-HV	6	ORF46	ORF34	UL96	U67	(65, 250)
U_L15		The ORF consists of two exons. The intron encodes a sequence antisense to U_L16 and U_L17 proteins. U_L15 is a 735-residue (M_r of 83,000) protein that binds U_L28 protein. U_L15 is required for cleavage and packaging of viral DNA and with U_L28 may function as a terminase. U_L15 is cleaved near the amino terminus, a reaction coupled with maturation of viral DNA into unit length molecules. γ_1.	α-HV, β-HV, γ-HV	6	ORF 42/45	ORF29a	UL89ex1	U66 ex1	(9, 64, 206, 208)
$U_L15.5$		$U_L15.5$ Exon 2 or U_L15 encodes a M_r 55,000 protein associated with capsids. 293 aa. Unknown	α-HV, β-HV, γ-HV	6		ORF29b	UL89ex2	U66ex2	(9)
U_L16		The 373-residue U_L16 protein is encoded in the intron located between exon 1 and 2 of U_L15. It is a virion component not essential for viral replication. γ_1.	α-HV, β-HV, γ-HV <positional>	6	ORF44	ORF33	UL94	U65	(174, 208)
UL17		The U_L17 ORF is contained in the intron located between exon 1 and 2 of U_L15, and encodes a 703-residue protein essential for DNA cleavage and packaging. In productive infection U_L17 localizes preformed capsid to the replication compartment. U_L17 has also been reported to be a tegument protein. γ_1.	α-HV, β-HV, γ-HV <positional>	6	ORF43	ORF32	UL93	U64	(206, 207, 227)
U_L18	VP23	The 318-residue (M_r 34,000) protein encoded by U_L18 is a capsid protein designated VP23. Together with the VP19C encoded by U_L38 it forms triplexes consisting of two copies of VP23 and one copy of VP19C. The 320 triplexes connect adjacent hexons (150) and pentons (12). γ_1.	α-HV, β-HV, γ-HV	5	ORF41	ORF26	UL85	U56	(124, 231)

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d]				Ref.
					VZV	γ-HV	HCMV	HHV-6	
U_L19	ICP5	Major capsid protein made of 1311 residues (M_r 149,000). Capsid hexons appear as tower like complexes containing 6 copies of the protein whereas pentons contain 5 copies. γ_1.	α-HV, β-HV, γ-HV	5	ORF40	ORF25	UL86	U57	(32, 175, 177, 243)
U_L20		U_L20 is a 222 residue polytopic membrane protein, with four predicted transmembrane segments. It localizes to virions, nuclear membranes and Golgi but is absent from plasma membranes. When expressed singly, it localizes mainly to endoplasmic reticulum. Coexpressed with gK, It localizes to the Golgi. Dispensable in cell culture. A deletion mutant in UL20 gene is detective in transport of the virions out of the perinuclear space, particularly in cells with fragmented Golgi. The ΔUL20 mutant is syncytial. Together with gK, inhibits cell-cell fusion. Its postulated role is to prevent fusion of the infected cells with adjacent cells. γ.	α-HV		ORF39				(5, 6, 13, 86)
$U_L20.5$		The $U_L20.5$ ORF is located 5′ to U_L20. The 160-residue protein localizes at small dense nuclear structures containing ICP22, $U_S1.5$, U_L3, and U_L4 proteins and is dispensable for viral replication. Its function is not known. γ_2.	None						(237)
U_L21		The 535-residue U_L21 protein is in tegument and may be weakly associated with capsids. U_L21 binds to microtubules but not to purified tubulin. In overexpressed cells it induces the formation of long cytoplasmic projections possibly due to its interaction with microtubules. It is not required for viral replication in cultured cells. γ_1.	α-HV		ORF38				(8, 226)
U_L22	gH	The 838-residues glycoprotein is essential for virion infectivity and cell-cell fusion. In cells infected with a deletion mutant virus in gH or a *ts* mutant at non permissive temperature, viruses lacking gH egress from the cell but are non infectious. Transport of gH from the endoplasmic reticulum to the Golgi and plasma membranes requires the interaction with the soluble gL. gH is an essential component of the complex enabling cell-cell fusion and induces neutralizing antibodies. VZV gH carries an endocytosis motif in the C-tail, absent from HSV gH. γ. Carries elements typical of viral fusion proteins. Possible fusogen.	α-HV, β-HV, γ-HV	4	ORF37	ORF22	UL75	U48	(36, 73, 79, 85, 94, 184)
U_L23	Thymidine kinase	Although known primarily as a thymidine kinase, it is a wide spectrum nucleoside kinase capable of phosphorylating both purine and pyrimidine nucleosides and their analogues. 376 aa. β.	α-HV, γ-HV		ORF36	ORF21			(63, 89, 158, 170)
U_L24		A 269-residue membrane-associated nuclear protein with an apparent M_r of 30,000. Mutations or deletions result in formation of small polykaryocytes in cell culture, somewhat diminished virus yields and decreased virulence in mice. The bulk of the protein is translated from the first AUG. A shorter transcript containing the second in frame AUG is also translated but the translation of 2 additional 3' co-terminal transcripts encoding C-terminal portions of U_L24 protein is uncertain. The UL24 ORF partly overlaps the TK gene in antisense direction. UL24 is dispensable in cell culture. γ_1.	α-HV, β-HV, γ-HV	4	ORF35	ORF20	UL76	U49	(185)

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d] VZV	γ-HV	HCMV	HHV-6	Ref.
UL25		The 580 residue product of the U_L25 ORF encodes a capsid protein involved in packaging of viral DNA into capsids. Dispensable for generation of the S terminus but required for correct generation of the L-terminus and possibly for retention of DNA in the capsid. γ_2.	α-HV, β-HV, γ-HV	4	ORF34	ORF19	UL77	U50	(161, 223)
U_L26	capsid scaffolding protein and protease	This ORF encodes a protein of 635 residues that is cleaved in *cis*- or *trans*- by itself at two sites. The N-terminal polypeptide is a protease required for assembly of a scaffolding within capsids for DNA packaging. The larger, middle cleavage product is a component of the scaffolding. γ.	α-HV, β-HV, γ-HV	4	ORF33	ORF17	UL80	U53	(90, 136, 137)
$U_L26.5$		The promoter and coding domain of $U_L26.5$ is contained in its entirety within the U_L26 ORF. The protein product of 328 residues is identical to the corresponding sequence of the U_L26 protein. It is cleaved by the protease at its C-terminus at the same site as the U_L26 protein. γ.	α-HV	4	ORF 33.5			U53.5	(137)
U_L27	gB	A 904-residue glycoprotein with apparent M_r of 110.000. It carries two, or three trasmembrane segments. Essential for virion infectivity and cell-cell fusion. Virions lacking gB egress the cell but are non infectious. The protein binds heparan sulfate and is a component of the cell-cell fusion complex. The cytoplasmic tail carries syncytial mutations, and endocytosis motifs, which mediate gB endocytosis into large vacuoles. Down modulation of gB at cell surfaces may be responsible for negative control of the cell-cell fusion. A *ts* mutation in ectodomain affects virus entry into the cell (*ts* B5). Induces neutralizing antibodies. γ_1. Crystal structure shows a trimer. Possible fusogen.	α-HV, β-HV, γ-HV	2	ORF31	ORF8	UL55	U39	(37–39, 109, 151, 186)
$U_L27.5$		The $U_L27.5$ ORF maps antisense to U_L27 and yields a γ_2 protein with an apparent M_r of 43,000. The predicted size of the coding capacity of the ORF is much larger. The protein accumulates in the cytoplasm. Its function is unknown. 575 aa. γ_2.	HSV-2						(46)
U_L28		A component of cleavage and packaging complex. See Functions of U_L15 and U_L6. 785 aa. Binds *pac1* sequences and is required for correct generation of the L-terminus. See functions of U_L6, U_L14 and U_L33. γ.	α-HV, β-HV, γ-HV	2	ORF30	ORF7	UL56	U40	(23)
U_L29	ICP8, single-stranded DNA binding protein	ICP8 is an essential 1196 residue single stranded DNA binding protein made very early in infection. ICP8 is required for viral DNA synthesis. β.	α-HV, β-HV, γ-HV	2	ORF29	ORF6	UL57	U41	(150, 253)
U_L30	DNA pol.	DNA polymerase. 1235 aa. γ.	α-HV, β-HV, γ-HV	2	ORF28	ORF9	UL54	U38	(29, 114, 234, 239, 240)
U_L31		Nuclear phosphoprotein that requires U_L34 protein for localization at the inner nuclear membrane. Binds lamin A/C in the nuclear lamina and directs envelopment at inner nuclear membrane. Carries a nucleotidylation recognition sequence. 306 aa. γ.	α-HV, β-HV, γ-HV	1	ORF27	ORF69	UL53	U37	(27, 48, 49, 147, 200, 201)
U_L32		U_L32 is 596-residue protein required for cleavage and packaging of viral DNA. The predominant localization of U_L32 in the cytoplasm suggests that either it shuttles between the nucleus and cytoplasm, or that it encodes additional functions. γ_2.	α-HV, β-HV, γ-HV	1	ORF26	ORF68	UL52	U36	(47, 130)

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d]				Ref.
					VZV	γ-HV	HCMV	HHV-6	
U_L33		A 130-residue capsid associated protein that forms a complex with the U_L15 and U_L28 proteins. Required for packaging of viral DNA into preformed capsids. γ_2.	α-HV, β-HV, γ-HV	1	ORF25	ORF67A	UL51	U35	(22, 199)
U_L34		An essential membrane-associated virion protein of 275 residues was reported to be a type 2 membrane protein phosphorylated by U_S3 kinase. Required for virion envelopment at inner nuclear membrane. Exclusive localization of UL34 protein at the nuclear membrane requires U_L31 protein. Extracellular virions do not contain UL31-UL34 proteins. The U_L34 homologue in murine cytomegalovirus recruits cellular protein kinase C to the nuclear lamina and induces lamin phosphorylation. γ_1.	α-HV, β-HV, γ-HV	1	ORF24	ORF67	UL50	U34	(200, 201, 215)
U_L35	VP26, capsid protein NC7	The 112-residue (M_r 12,000) protein forms a hexameric structure located on the outer surface of each hexon. Previously know as NC7. Dispensable for growth in cell culture but not in vivo. γ_2.	α-HV, β-HV, γ-HV	1	ORF23	ORF65	UL49	U32	(57, 71)
U_L36	VP1/2	The 3165-residue (M_r 270,000) protein essential for viral replication is located in the tegument essential for egress of virions through the cytoplasm. A *ts* mutation in UL36 blocks release of viral DNA into the nucleus at the non permissive temperature. γ_2.	α-HV, β-HV, γ-HV	1	ORF22	ORF64	UL48	U31	(20, 72)
U_L37	tegument protein	A 1123-residue (M_r 120,000) tegument phosphoprotein binds to DNA in the presence of ICP8. It is essential for viral replication. In its absence, nucleocapsids accumulate aberrantly in the nucleus and unenveloped capsids accumulate in the cytoplasm. γ_1.	α-HV, β-HV, γ-HV	1	ORF21	ORF63	UL47	U30	(146, 213, 247)
U_L38	VP19C Capsid protein	This 465-residue protein forms triplexes together with VP23, consisting of two copies of VP23 and one copy of VP19C. The 320 triplexes connect adjacent hexons (150) and pentons. γ_2.	α-HV	1	ORF20	ORF62	UL46	U29	(219, 231)
U_L39	Ribonucleotide reductase, large subunit	This ORF encodes a 1137-residue protein of M_r of 136,000. The protein is anchored in membranes and has protein kinase activity mapping to the N terminus but not required for ribonucleotide kinase activity. U_L39 may play a role in maintaining dTTP pools in infected cells. β.	α-HV, β-HV, γ-HV	1	ORF19	ORF61	UL45	U28	(54, 68, 189)
U_L40	Ribonucleotide-reductase small subunit	This ORF encodes the 340-residue small subunit of ribonucleotide kinase. β.	α-HV, γ-HV		ORF18	ORF60			(108)
U_L41	vhs, virion host shutoff protein	*vhs* (virion host shutoff protein) is a 489-residue protein packaged in the tegument and mediates the degradation of RNA early in infection. The degradation appears to be selective for viral RNAs. At late times after infection it is associated with VP16 (product of U_L48) and no longer exhibits this function. γ_1.	α-HV		ORF17				(82, 83, 211, 216, 225)
U_L42		DNA polymerase accessory protein. Binds double stranded DNA. It also associates with cdc2 and topoisomerase IIa. 488 aa. β.	α-HV, β-HV, γ-HV	1	ORF16	ORF59	UL44	U27	(3, 96)
U_L43		Non-essential protein. Sequence suggests that it is a hydrophobic, myristylated integral membrane protein. 434 residues. γ.	α-HV		ORF15				(44, 144, 145)

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d]				Ref.
					VZV	γ-HV	HCMV	HHV-6	
U_L43.5		This ORF is located antisense to U_L43. Tagged epitope revealed the synthesis of a M_r 32,000 γ_2 protein, dispensable for growth in cell culture. Its function is not known. γ.	HSV-2						(44, 236)
U_L44	gC	A 511 residue glycoprotein heavily N- and O-glycosylated. Some gC-minus mutants arise in culture, but all primary isolates express gC. A deletion gC mutant attaches to cells with reduced efficiency, but is viable. gC mediates attachment of virions to glycosaminoglycans of heparan sulphate, or chondroitin sulphate. The heparan sulphate binding site on gC maps to the N-terminus of the ectodomain. gC is part of the immune evasion strategy of HSV, as it carries two domains involved in modulating complement activation; one binds C3, and the other is required for blocking C5 and properdin binding to C3. Each region contributes to virulence, as viruses lacking these domains are less virulent than wt-virus. γ_2.	α-HV		ORF14				(88, 103, 122, 142)
U_L45		The 172 residue type 2 membrane protein is dispensable for viral replication in cultured cells. U_L45 is required for herpes simplex virus type 1 glycoprotein B-induced fusion. It is dispensable for growth in cell culture. γ_2.	HSV-2, B virus						(98, 233)
U_L46	VP11/12 tegument protein,	The ORF encodes the 718-residue tegument proteins VP11 and VP12, differentiated solely by their migration in denaturing gels. The ORF is dispensable in cultured cells. Available data suggests that it is a γ_1 protein present along with U_L47 in stechiometric amounts with U_L48 gene product and capable of enhancing its activity. In the absence of U_L48 protein, U_L46 protein inhibits activation of α promoters. γ.	α-HV		ORF12				(157, 252)
U_L47	VP13/14 tegument protein	The two products of the ORF have apparent M_r of 82000 and 81000. They are abundant glycosylated, phosphorylated components of the tegument, dispensable for viral replication in cells in culture. U_L47 binds RNA and is reported to shuttle between the cytoplasm and nucleus. 693 aa. γ_1.	α-HV		ORF11				(77, 123, 164)
U_L48	α-TIF, VP16, ICP25	α-TIF (α-trans-inducing factor), a 491 residue protein, is a multifunctional tegument protein. It induces α-genes by interacting with two cellular proteins, HCF and Oct-1. The complex binds to specific sequences with the consensus GyATGnTAATGArATTCyTTGnGGG-NC. It is also required for virion assembly. Essential for growth in cell culture and has gamma expression regulation. Crystal structure of the conserved core has been solved. γ.	α-HV		ORF10				(59, 138, 172, 187)
U_L49	VP22	Encodes the nonessential tegument protein VP22. VP22 translocates into cells exposed to the protein. Prior to cell division it localizes to microtubules in the cytoplasm. After cell division it is bound to chromatin. VP22 binds RNA and is thought to translocate mRNA from infected to uninfected cells. It has also been reported to bind membranes, and to induce the stabilization and hyperacetylation of microtubules (putative microtubule-associated protein). It is not required for tegument assembly and its role in viral replication is unclear. γ.	α-HV		ORF 9				(33, 80, 194, 214)

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d]				Ref.
					VZV	γ-HV	HCMV	HHV-6	
U_L49.5	gN	This 91 residues membrane-associated protein with apparent M_r of 6700 is abundant in virions. The PrV homologue is gN, which forms a complex with gM. In other herpesviruses the complex is gM-gN-gO. PrV gN is not accessible in the infected cell plasma membrane. In other herpesviruses, it forms a complex with gM that inhibits fusion. In PrV, gN may be disulphide-linked to the tegument, and gN was absent from gM-negative PrVirions, whereas gM was readily detected in virions in the absence of gN. Thus, gM appears to be required for virion localization of gN. UL49.5 is dispensable for viral replication in cultured cells. γ2.	α-HV, β-HV, γ-HV						(2, 17, 19, 134)
U_L50	deoxyuridine triphosphatase, dUTPase	U_L50 encodes a dUTPase. The ORF is not essential for viral replication in cells in culture. 371 aa. β.	α-HV, β-HV, γ-HV		ORF 8				(14, 17, 205)
U_L51		The 244 residue protein is a component of the virion tegument. Dispensable for growth in cell culture. γ_1.	α-HV, β-HV, γ-HV		ORF 7				(17, 66)
U_L52		U_L52 is a component of the helicase-primase complex. It strongest affinity appears to be for ICP8, but both U_L52 an U_L5 proteins are required for DNA binding. 1058 aa. β.	α-HV, β-HV, γ-HV		ORF6				(26, 62)
U_L53	gK	The 338 amino acid protein encoded by this ORF is a low abundance glycoprotein with M_r 40000. Sequence predicts three or four transmembrane segments. Topology, investigated by epitope mapping, shows an extracellular N-terminus and intracellular C-terminus. In infected cells gK localizes to Golgi apparatus. Plasma membrane localization is debated. gK is the most frequent locus of syncytial (syn) mutations. A deletion mutant in gK is defective in exocytosis of virions, and is syncytial. When expressed singly, gK localizes mainly at ER. Its transport to the Golgi apparatus requires the UL20 protein. gK inhibits fusion in the cell-cell fusion assay. Inhibition is augmented by coexpression with UL20 protein. gK role appears to prevent infected cells from fusing with adjacent cells. γ.	α-HV		ORF5				(4, 5, 70, 87, 107, 167, 193)
U_L54 α27	ICP27	ICP27 is a multifunctional 512-residue protein with two well defined functions. Early in infection it blocks splicing of RNA, thereby enabling the transport of unspliced RNA into the cytoplasm. At late times it acts as an RNA transporter and shuttles between the nucleus and cytoplasm. α.	α-HV, β-HV, γ-HV		ORF4	ORF57	UL69	U42	(21, 51, 52, 173, 210, 220)
U_L55		This ORF encodes a nonstructural 186-residue protein associated with sites of virion assembly. It is dispensable for viral replication in cultured cells. γ_2.	α-HV (not PrV)		ORF 3				

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d] VZV	γ-HV	HCMV	HHV-6	Ref.
U_L56		This ORF encodes a phosphorylated 234-residue, type II membrane protein associated with virions. The protein is essential for pathogenesis in experimental animal systems but is not required for viral replication in cultured cells. γ_2	HSV-2						(127, 204)
α4	ICP4	ICP4 is an essential regulatory 1298-residue protein. It acts as a transactivator and a repressor of viral gene functions. As a repressor it binds to high affinity sites overlapping the transcription initiation sites of its own ORF and that of ORF P and ORF O. The interaction of ICP4 together with transcriptional factors to low affinity sites -some significantly divergent from the high affinity sites – may account for the trans activating function of ICP4. While the interaction of ICP4 with transcriptional factors has been well documented, the role of low affinity sites remains unclear. α.	α-HV		ORF62/71				(61, 97, 121, 183, 188)
α 22 U_S1	ICP22	This ORF encodes a 420-residue regulatory protein of 420 residues essential in some cells and in experimental animal systems but not in human or primate cells in continuous cultivation. See $U_S1.5$ for details regarding its functions. The protein is extensively phosphorylated by viral and cellular kinases and nucleotidylylated by casein kinase II. α.	α-HV		ORF63/70				(3, 7, 31, 118, 139, 165, 195, 209)
$U_S1.5$		The promoter and coding domain of $U_S1.5$ is contained within the α22 ORF and the α22 met 14 acts as the initiator methionine of $U_S1.5$ protein. Most of the known functions of ICP22 map to the $U_S1.5$ ORF. Thus in the absence of ICP22 or U_L13 a set of late proteins (U_L41, U_S11 and U_L38) accumulates in smaller amounts. The results of two studies may account for this observation. Thus, both ICP22 and U_L13 are required for the activation of cdc2 and degradation of the partners, cyclin A and B. cdc2 partners with U_L42 and together bind and mediate posttranscriptional modification of topoisomerase IIα. In other studies the two proteins have been shown to mediate an "intermediate" phopshorylation of RNA Polymerase II. ICP22 also affects the accumulation of α0 mRNA. 274 aa. VZV ORF63/70 appears to correspond to Us1.5 rather than to ORF Us1. α.	α-HV		ORF63/ 70				(3, 43)
U_S2		The ORF encodes a 291 residue tegument protein not essential for viral replication in culture or in experimental animals. It is conserved among most α-HV. Its function is not known. The PrV homologue is prenylated. γ_2.	HSV-2 and B virus, but not PrV or VZV						(55, 112, 163)
U_S3	Serine/threonine protein kinase	A multifunctional protein kinase that phosphorylates a large number of both cellular and viral proteins. The kinase is not essential for viral replication. U_s3 protein kinase blocks apoptosis induced by viral mutants or exogenous agents. 481 aa. γ_1.	α-HV		ORF66				(24, 102, 119, 120, 133, 197)

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d]				Ref.
					VZV	γ-HV	HCMV	HHV-6	
U_S4	gG	A 238 residue envelope glycoprotein of unknown function used in serologic assays to differentiate HSV-1 from HSV-2 antibody responses. The HSV-2 gG is larger that HSV-1 gG. γ_1.	α-HV except VZV						(1, 131)
U_S5	gJ	This 92-residue glycoprotein protects the cells from apoptosis induced by gD−/+ and gD−/− virions. Dispensable in cell cultures. γ.	HSV-2, B virus, not PrV or VZV						(111, 254)
U_S6	gD	gD is a virion glycoprotein of 394 residues, with apparent M_r of 56000, essential for virus entry into the cell, and cell-cell fusion. gD is the receptor-binding glycoprotein, and interacts with three alternative receptors, named HVEM, nectin, and modified heparan sulphate. Major determinant of HSV tropism. Crystal structure shows an Ig-folded core with N- and C-terminal extensions. Upon receptor binding, a change in conformation ensues. A deletion gD mutant produces virions that exit the cell, but are non infectious. Soluble gD blocks infectivity. Ectopic expression of full length gD induces in the cell restriction to infection, by sequestering the gD receptor. Mutations at N-terminus allow usage of an alternative receptor. gD protects the cell from apoptosis induced by gD−/+ and gD−/− virions. Antiapoptotic activity is mediated by mannose-phosphate receptor. β-γ.	α-HV except VZV						(42, 53, 56, 92, 135, 180, 254)
U_S7	gI	gI is a 390-residue virion glycoprotein. It forms a heterodimer with gE. The gI-gE complex constitutes a viral Fc receptor for monomeric IgG. The gE-gI complex has basolateral localization in epithelial cells and facilitates basolateral spread of progeny virus in polarized cells. Dispensable in transformed cells, but critical in non transformed cells. γ_1.	α-HV		ORF67				(74, 75, 149, 168, 235)
U_S8	gE	gE is a 550-residue virion glycoprotein. It forms a heterodimer with gI. See, gI. gE is phosphorylated by UL13 protein kinase. γ_1.	α-HV		ORF68				(74, 75, 166, 182, 255)
$U_S8.5$		The 159-residue (M_r 19,000) protein localizes to nucleoli and is dispensable for viral replication. Its mRNA is among the most abundant species packaged in virions. γ_1.	HSV-2, B virus, not PrV or VZV						(91, 214)
U_S9		A dispensable type II membrane-associated protein of 90 residues, reported to play no role in neurovirulence or latency. May be involved in anterograde axonal transport of virions. γ.	α-HV		ORF65				(34, 58, 178, 179)
U_S10		A dispensable tegument phosphoprotein made of 312 residues. Reported to play no role in neurovirulence or latency and to copurify with the nuclear matrix. γ_1.	α-HV		ORF 64/69				(179, 218, 248)
U_S11		This ORF encodes an abundant 161-residues protein expressing multiple functions. Virion associated U_S11 localizes to polyribosomes. Late in infection U_S11 is also found in nucleoli. $U_S11$synthesized under an α promoter blocks phosphorylation of eIF-2α. U_S11 binds RNA in sequence and conformation dependent fashion and is in part responsible for the packaging of RNA in virions. γ_2.	HSV-2						(45, 202, 214)

(*cont.*)

Gene designation	Alternative name	Main properties	Conservation[b]	Gene Block[c]	Homologues[d] VZV	γ-HV	HCMV	HHV-6	Ref.
U_S12, α47	ICP47	The small 88-residues ORF encodes a M_r 9776 protein that binds to TAP1/TAP2 and blocks the transport of antigenic peptides to ER for presentation by MHC class 2 proteins. α.	HSV-2, B virus, not PrV or VZV						(104, 155, 156, 251)
LAT		The primary latency associated transcript is a low abundance transcript 8.5 to 9 kb in size and extends through the length of the ab and b'a' sequences flanking the unique long sequences. Both productively infected and latently infected cells accumulate transcripts 2.0 and 1.5 kb in size thought to be stable introns of the primary transcript. The smaller LAT sequences terminate antisense to and within the coding sequences of α0 gene. Deletion of the sequences encoding LAT reduces the mortality and morbidity in experimental animal systems and the number of neurons harboring latent virus. The decrease in the number of neurons in LAT minus mutants has been linked to pro-apoptotic manifestations of latent virus. Pre-α.							(191, 221, 246)
ORI_S RNA		This RNA originates in the **c** sequences flanking the unique short sequences at or near the transcription initiation sites of α22 or α47 genes, extends across the ORI_S sequence and co-terminates with the transcript encoding ICP4. The RNA is detected after the onset of viral DNA synthesis. Its function is not known.							(105)
αX and βX RNAs		These overlapping RNAs 0.9 and 4.9 Kb in size originate upstream of ORF P and extend across the L-S junction. Their function is not known.							(30)
AL-RNA		RNA reported to be antisense to the 5' sequence of LAT							(192)
VZV ORF1		Membrane protein			ORF1				(60)
VZV ORF 2					ORF2				(212)
VZV ORF 13		Thymidylate synthetase			ORF 13				
VZV ORF 32					ORF 32				
VZV ORF 57					ORF 57				
VZV ORFS/L					ORF S/L				(115)

[a] kinetic class: α, β, γ, Unknown.
[b] Abbreviations: alphaherpesvirinae or α-HV, betaherpesvirinae or β-HV, gammaherpesvirinae or γ-HV
[c] Conserved gene block to which the sequences belongs
[d] Identification of homologues in other herperviruses was based on informations contained in ref.s 69, 95, 125, 162, 169, 190, 228

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7

Entry of alphaherpesviruses into the cell

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Introduction

Herpes simplex virus (HSV) represents the most comprehensive example of virus-receptor interaction in the *Herpesviridae* family, and the prototype virus encoding multipartite entry genes. Whereas small enveloped viruses package the functions required for entry and fusion into one or two fusion glycoproteins, in HSV the same functions are distributed over several distinct glycoproteins, each with a specialized activity. In addition, HSV encodes a highly sophisticated system for promoting and blocking fusion between the viral envelope and cell membrane. Because the most obvious models of virus entry into the cell do not fit with the HSV complexity, and despite our detailed knowledge of the HSV receptors and of the crystal structure of glycoprotein D (gD), the receptor-binding glycoprotein, and of gB, HSV entry is still, in part, a puzzle (WuDunn and Spear, 1989; Cocchi *et al.*, 1998b; Geraghty *et al.*, 1998; Carfi *et al.*, 2001).

The current model of HSV entry envisions that, first, the virus attaches to cell membranes by the interaction of gC, and possibly gB, to glycosaminoglycans (GAGs) (Herold *et al.*, 1991). This binding likely creates multiple points of adhesion, is reversible, and the detached virus maintains its infectivity, indicating that fusion has yet to take place. Penetration requires gD, whose ectodomain contains two physically separate and functionally distinct regions, i.e., the region made of the N-terminus that carries the receptor-binding sites, and the C-terminus that carries the profusion domain (Ligas and Johnson, 1988; Cocchi *et al.*, 2004). The role of gD in entry is to interact with one of the entry receptors, to signal receptor-recognition and thus trigger fusion, by recruiting three additional glycoproteins – gB, gH, gL. The trio of gB, gH, gL, execute fusion with the plasma membrane or endocytic vesicle of the target cell (Fig. 7.1) (Cai, W. H. *et al.*, 1988; Forrester *et al.*, 1992; Roop *et al.*, 1993; Campadelli-Fiume *et al.*, 2000; Spear *et al.*, 2000; Nicola *et al.*, 2003). Of these, gH carries elements characteristic of viral fusion glycoproteins, i.e., a hydrophobic α-helix with attributes of an internal fusion peptide, and two heptad repeats with propensity to form coiled coils (Gianni *et al.*, 2005a,b). gB is a trimer with a coiled coil core; its structure closely resembles that of viral fusion proteins (Heldwein *et al.*, 2006; Roche *et al.*, 2006). Following fusion, the released tegumented nucleocapsid travels along microtubules to the nuclear pore, where the viral DNA is released into the nucleus (Sodeik *et al.*, 1997).

Much less is known about varicella zoster virus (VZV) entry. The process may be very different from that of HSV inasmuch as virion-to-cell infection is inefficient in the VZV system, and the viral genome presents a striking difference from that of HSV, namely the lack of gD (Davison and Scott, 1986).

The membrane proteins

The HSV envelope contains at least eleven glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM). Additional membrane proteins not detected in the extracellular virion envelope are UL20, UL34, UL45 and possibly US9. The transcripts for UL24, UL43, and UL49.5 ORFs have been recognized, but the proteins have yet to be identified. A summary list with references is presented in Chapter 6.

From a structural point of view, the majority of HSV glycoproteins are type I glycoproteins. Variants include gL, which is soluble (Hutchinson *et al.*, 1992a); gB, which may carry two or three α-helices in the transmembrane (TM) region (Pellett *et al.*, 1985) and gK, gM, and UL20, which carry information for multiple transmembrane segments

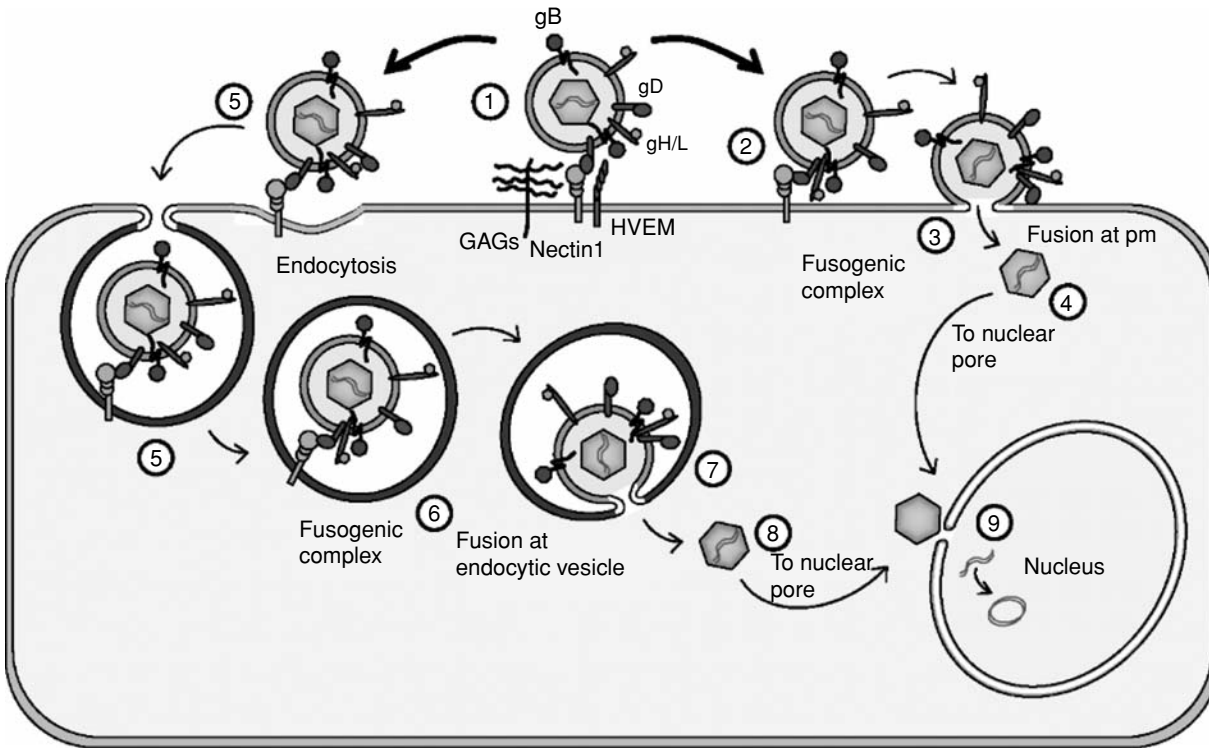

Fig. 7.1. Schematic drawing of HSV entry. Entry can occur either by endocytosis (pathway at the left), or by fusion at the plasma membrane (pathway at the right). Following attachment to cells, gD binds to a cellular receptor (frame 1), and presumably following a conformational change it recruits the glycoproteins B, H and L in an active fusogenic complex (frame 2), triggering the fusion between viral envelope and cellular plasma membrane (frame 3). The naked nucleocapsids are transported to the nucleus (frames 4 and 9). In a cell line-dependent manner or with modified forms of the receptor (see text for details) bound virions can enter cells by endocytosis (frame 5). It is conceivable that at this stage the four fusogenic glycoproteins are in a non-fusion-active form. Following acidification/maturation of the endocytic vesicles, a fusogenic complex may form (frame 6) and fusion ensue between the virion envelope and the vesicle membrane (frame 7). Nucleocapsids delivered to the cytoplasm are transported to the nucleus (frames 8 and 9).

(McGeoch *et al.*, 1988). US9 and HSV-2 UL45 are type II glycoproteins with a C-terminal ectodomain.

At the ultrastructural level, HSV glycoproteins form long thin spikes, each made of a single species. As visualized in cryo-electron tomograms of isolated virions, the envelope contains 600–750 glycoprotein spikes that vary in length, spacings, and in the angle at which they emerge from the membrane. Their distribution in the envelope suggests functional clustering (Grunewald *et al.*, 2003). In contrast, slender spikes have not been seen in varicella zoster virions (VZV) grown in cultured cells. Instead, the virion appears to be covered by an envelope studded with protrusions rather than spikes. An example is shown in Fig. 7.2. Further studies at even higher resolution will be required to determine differences between HSV and VZV envelopes.

HSV

Attachment to cells

Attachment of HSV to cells occurs upon binding of gC to GAGs that decorate heparan sulphate or chondroitin sulphate (Spear *et al.*, 1992) (Fig. 7.1). This step enhances HSV infectivity, but is not an absolute requirement, as cells defective in heparan sulphate and chondroitin sulphate exhibit a 100-fold reduced susceptibility to infection, yet can be infected (Gruenheid *et al.*, 1993). A large variety of viruses use heparan sulphate proteoglycans as receptors; their broad expression argues that they can not be responsible for any specific viral tropism.

The major actor during attachment is gC, a non essential glycoprotein encoded by the UL44 gene. gC is a

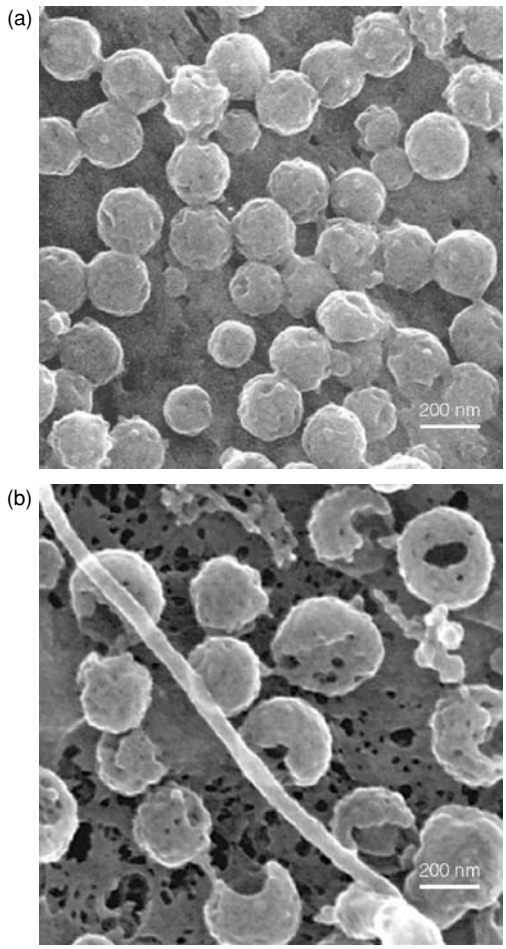

Fig. 7.2. Scanning electron micrographs of HSV-1 and VZV. Images of both viruses were taken by SEM after infection of cultured cells. HSV-1 virions have a more uniform appearance although indentations are seen in an occasional virion envelope (a). In contrast, VZV virions are more aberrant (b). Several of the virions have indentations, while other virions have incomplete envelopes. Since both viruses were examined under the same SEM conditions, it is unlikely that the aberrant nature of the VZV envelope is due to fixation artifacts. Micrographs kindly provided by Dr. Charles Grose.

mucin-type glycoprotein because of its high content in N-linked and O-linked oligosaccharides. Its ectodomain structure is provided in part by 8 cysteines, and harbors two physically separate antigenic regions, antigenic sites I and II, that map at the C- and N-termini of the molecule, respectively (Dolter *et al.*, 1992). Evidence for the role of gC in attachment rests on several lines of evidence. Initially, it was observed that the polycations neomycin and polylysine inhibit attachment of HSV-1, but not HSV-2 to cells, and this differential effect was mapped to gC (Campadelli-Fiume *et al.*, 1990). gC and gB bind heparin-Sepharose columns (Herold *et al.*, 1991). The affinity of binding to heparan sulphate is on the order of 10^{-8} M (Rux *et al.*, 2002). The region important for the interaction with heparan sulphate maps to the N-terminus of gC (Tal-Singer *et al.*, 1995). Virion binding to cells is reduced in HSV-1 mutants lacking gC-type1 gene, but not in HSV-2 mutants lacking gC-type2 (Herold *et al.*, 1991; Gerber *et al.*, 1995). The majority of these studies were performed with mutants constructed in the background of the HSV-1(KOS) strain. In contrast, deletion of gC gene in the genetic background of Sc16 and HFEM strains yielded viruses with unimpaired attachment activity, suggesting that in different virus strains, attachment may be carried out by different proteins (Griffiths *et al.*, 1998). gC has also been implicated in virus attachment to the baso-lateral domain of MDCK polarized epithelial cells, and to the apical domain of polarized human CaCo2 cells.

Interaction of gD with its receptors

gD

The ectodomain of gD is required and sufficient to enable HSV entry into cells. It is made of two separate and distinct regions, i.e., the N-terminus, carrying the receptor-binding sites (approximately contained between residues 1 and 250–260), and the C-terminus carrying the profusion domain (residues 250–260 to 305) (Cocchi *et al.*, 2004).

A breakthrough in our understanding of HSV entry came from resolution of the crystal structure of a soluble form of gD, initially up to amino acid residue 259 (Carfi *et al.*, 2001), and later on up to residue 306 (Krummenacher *et al.*, 2005). The initial structure was determined for gD alone and for gD in complex with one of the gD receptors, HVEM (herpesvirus entry mediator) (Fig. 7.3). The N-terminus consists of three portions, an Ig-folded central core (residues 56–184) made of β-strands forming two antiparallel β-sheets, and two extensions, one N-terminal (residues 1–37) and one C-terminal (Fig. 7.3). The N-terminus, which harbors all the contact residues to HVEM, is disordered in the crystal of gD alone, but forms a hairpin when gD is complexed with HVEM. gD and HVEM thereby form an intermolecular β-sheet, which is believed to stabilize the complex. Formation of the N-terminal hairpin documents a conformational change to gD when it binds HVEM.

The crystal structure of gD in complex with nectin1 has not yet been solved. The nectin1-binding site on gD, determined by means of insertion-deletion or substitution mutants, appears to be more widespread than the HVEM interaction region, (Milne *et al.*, 2003; Yoon *et al.*, 2003; Zhou

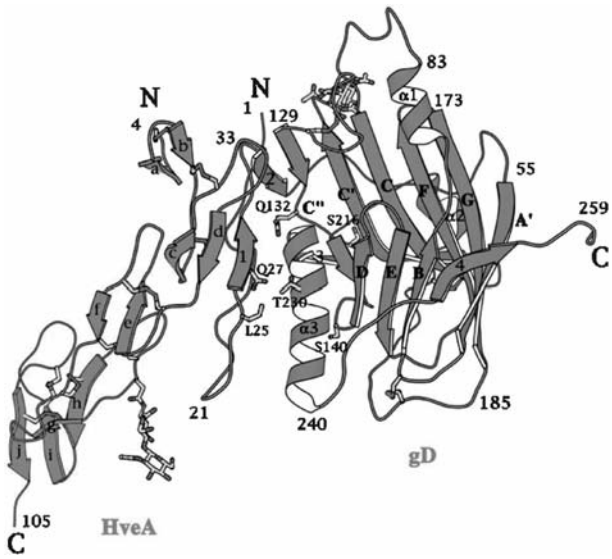

Fig. 7.3. Ribbon diagram of the 3D structure of a soluble truncated form of gD (gD285t, colored in orange) bound to HVEM receptor (HveA, colored in green) as determined by X-ray crystallography. The N-terminus (residues 1–37) of gD is devoid of a specific structure when in the unbound state, but folds into a hairpin when bound to HVEM receptor. The β-strand formed by residues 27–29 (indicated with number 1) forms an intermolecular β-sheet with HVEM residues 35–37 (letter d). The core of gD (residues 56–184) has a V-type immunglobulin domain structure, composed of 9 parallel and antiparallel β-strands (letters A to G) that form two opposing β-sheets, and carries an additional α-helix (α1). The residues 185–259 form two α-helices that fold back to the N-ter (α2 and α3), and two β-strands (numbered 3 and 4). The α3 helix supports gD's N-terminal hairpin. An additional β-strand (number 2) is located in the connector sequence (residues 33–55) that precedes the Ig-like core. Reprinted from (Carfi *et al.*, 2001), with permission. (See color plate section.)

et al., 2003; Jogger *et al.*, 2004; Manoj *et al.*, 2004; Connolly *et al.*, 2005). The only recombinant described so far debilitated for interaction with nectin1 carries the V34S substitution (Zhou and Roizman, 2006).

Receptors

Entry receptors interact with gD. This notion was established long before the actual receptors were identified, and rests on two lines of evidence. First, soluble gD binds in a saturable manner to cells and prevents infection (Johnson, D. C. *et al.*, 1990; Nicola *et al.*, 1997). Second, expression of gD from a transgene renders cells resistant to infection, because of its ability to sequester the receptor, a phenomenon designated restriction to infection or gD-mediated interference (Campadelli-Fiume *et al.*, 1988; Johnson, R. M. and Spear, 1989).

The search for HSV receptors was an active field in the 1990s, and a number of molecules were described as potential receptors. A breakthrough came from Spear and coworkers, who made use of HSV-resistant CHO cells, and identified a HeLa cell cDNA clone that encoded HVEM (Montgomery *et al.*, 1996). However, three observations in that study suggested the existence of additional receptors. First, HVEM appeared to be expressed by a limited number of cell lines. Second, antibodies to HVEM failed to completely block HSV infection. Third, several virus strains were unable to enter CHO cells expressing HVEM but were otherwise viable. This boosted further efforts in the field, which quickly led to the discovery of nectins, and, later on, of modified heparan sulphate. Altogether, the receptors known to date belong to three unrelated molecular families. Their present and past nomenclature, and the viruses for which they serve as receptors are reported in the table.

Nectins are intercellular adhesion molecules

Research in the field of nectins has proceeded in parallel with their characterization as HSV receptors (Takai *et al.*, 2003). Nectins 1–4 form a subfamily of Ca^{2+}-independent immunoglobulin (Ig)-type intercellular adhesion molecules. Together with nectin-like molecules and poliovirus receptor, they share the same overall structure consisting of three Ig-type domains. Splice variant isoforms are designated with Greek letters. Nectins form homo *cis*-dimers on the plasma membranes and *trans*-dimers with nectins present on the adjacent cell. Each nectin has a specialized pattern of *trans*-dimer formation with itself or other nectins (Reymond *et al.*, 2000; Takai *et al.*, 2003). Their main attribute is the formation, together with cadherins, of the adherens junctions of epithelial cells, and in cooperation or not with cadherins, the organization of claudin-based tight junctions. In addition, they are involved in the formation of synapses in neurons and the organization of heterotypic junctions between Sertoli cells and spermatids in the testis (Takai *et al.*, 2003).

Most nectins carry a C-terminal conserved motif that binds afadin, thus anchoring the adhesion molecules to the cytoskeleton (Takai *et al.*, 2003). This domain is absent from nectin1β which is not restricted to adherens junctions. Nectin-mediated signalling activity leads to activation of a variety of extracellular and intracellular molecules, such as scatter factor/hepatocyte growth factor, Ras, Cdc42 and Rac small G proteins (Takai *et al.*, 2003).

Table 7.1. Human HSV receptors and the viral strains for which they serve

		Human α-herpesvirus				
		Unrestricted/*rid*			Animal α-herpesvirus	
Receptor name	Alternative name	HSV-1	HSV-2	mutants	PrV	BHV-1
HVEM	HveA	+	+	−	−	−
Nectin1	PRR1, HIgR, HveC	+	+	+	+	+
Nectin2	PRR2, HveB	−	+/−	+	+	−

Nectin1

Human nectin1 is a broad spectrum receptor for human and animal alphaherpesviruses (Table 7.1). Three isoforms are known, two of which: -α and -β are membrane-bound (Cocchi *et al.*, 1998a,b; Geraghty *et al.*, 1998; Krummenacher *et al.*, 1998; Krummenacher *et al.*, 1999; Campadelli-Fiume *et al.*, 2000). Their main properties as HSV receptors are as follows.

(i) Nectin1 is broadly expressed in human tissues, including tissues and organs targeted by HSV, like CNS, ganglia and muco-epithelia (Cocchi *et al.*, 1998b; Haarr *et al.*, 2001; Matsushima *et al.*, 2003; Richart *et al.*, 2003; Linehan *et al.*, 2004). It is expressed in virtually all human cell lines, including epithelial cells, neurons and fibroblasts (Campadelli-Fiume *et al.*, 2000; Spear *et al.*, 2000). Some of these cells simultaneously express HVEM (Krummenacher *et al.*, 2004).

When HSV initially infects mucosal epithelium, the apical domain of polarized epithelial cells are targeted initially, whereas basolateral domains of epithelial cells are available to the virus only if a lesion disrupts the integrity of the lining. It was therefore of interest to know whether nectin1 can serve as an HSV receptor in polarized epithelial cells. Human CaCo2 cells can be infected with HSV from the apical domain (Griffiths *et al.*, 1998), whereas MDCK cells and primary human keratinocytes are preferentially infected from the basolateral domain (Schelhaas *et al.*, 2003; Marozin *et al.*, 2004). These differences may reflect cell line-dependent differences in the pattern of polarization of a same molecule, or distribution of receptors like nectin1-β and HVEM, which do not appear to be restricted to adherens junctions.

(ii) Nectin1 interacts physically with gD (Krummenacher *et al.*, 1999). The interaction requires the first 250 residues of gD and the V domain of nectin1. The affinity ranges from 10^{-6} to 10^{-8} molar, with the highest affinity observed with forms of gD that were truncated at or after residue 250. Affinity decreases by 100-fold with gD_{306t}, reflecting a folding of the most C-terminal portion of gD towards the core (Whitbeck *et al.*, 1999; Krummenacher *et al.*, 2005). Insertion mutations alter the binding affinity; insertions at the N-terminus (e.g., at residues 34, and 43) modify the binding to HVEM but not to nectin1 (Milne *et al.*, 2003; Jogger *et al.*, 2004). Remarkably, even when the binding affinities are low, or undetectable, the mutant forms of gD maintain the ability to mediate HSV entry and cell–cell fusion, implying that gD functions in virus entry and cell fusion regardless of its receptor-binding affinity and kinetics, and that as long as interaction with a functional receptor occurs, entry takes place (Milne *et al.*, 2003; Zhou *et al.*, 2003).

Human nectin1 also binds isoforms of gD from animal α-herpesviruses. The affinity may be even higher than for HSV-1 gD (in the case of PrV), or very low (in the case of BHV-1) (Connolly *et al.*, 2001). Even when the affinity is very low, human nectin1 is capable of mediating entry (Cocchi *et al.*, 1998b; Geraghty *et al.*, 1998).

(iii) The domain of nectin1 functional in HSV entry and in binding to gD was initially mapped to the N-terminal V domain, and subsequently to the C-C′-C″ ridge (Krummenacher *et al.*, 2000; Cocchi *et al.*, 2001; Menotti *et al.*, 2002b). Critical residues that may be part of the interface with gD are amino acids 77 and 85 (Martinez and Spear, 2002).

(iv) Nectin1-γ is a natural soluble isoform of nectin1 generated by alternative splicing. Although it contains the three Ig domains, it has a narrow distribution in human tissues, unlike nectin1-α and -β. Like soluble recombinant forms of nectin1, it has the capacity to bind to virions and block infectivity. An unexpected property was that the soluble nectin1-γ molecules suffices to mediate virus entry into receptor-negative cells. This may be consequent either to an association to endogenous nectins, or to a direct binding of the soluble receptor to virions (Lopez *et al.*, 2001).

Nectin 2 and the unrestricted or rid *mutations*

The remarkable feature about nectin2 is that a single amino acid substitution in gD confers to HSV the ability to use nectin2 as an alternative receptor, without hampering its ability to use nectin1 (Table 7.1). At the same time, this mutation abolishes the interaction with HVEM (Connolly *et al.*, 2003; Yoon *et al.*, 2003). The end result is that the

host range of the virus is modified. The mutations are L25P, Q27P, or Q27R, and are present in the unrestricted, or *rid* HSV-1 mutants. Nectin2 also serves as a weak receptor for some strains of HSV-2, but is inactive for wt-HSV-1 (Warner *et al.*, 1998; Lopez *et al.*, 2000; Krummenacher *et al.*, 2004). Physical interaction studies were in agreement with these properties (Warner *et al.*, 1998; Lopez *et al.*, 2000; Yoon *et al.*, 2003). The nectin2 residues critical for HSV entry were identified as amino acids 75–81 and 89, which lie adjacent to the predicted C′C″ β-strands, i.e., the region corresponding to the nectin1 region involved in interaction with wild-type gD.

The TNF receptor family

TNFRs (tumor necrosis factor receptors) form a family of signal transduction molecules involved in regulation of cell proliferation, differentiation and apoptotic death. Structurally, their ectodomain is composed of four typical cysteine-rich domains (CRDs). The family includes twenty nine human members, classified into three groups according to their cytoplasmic sequences and signaling properties. Members of the first group (exemplified by Fas) contain a death domain (DD) in the cytoplasmic tail. After binding to their ligands they interact with intracellular adaptors, which, in turn, induce apoptosis by activation of the caspase cascade. Members of the second group (exemplified by TNFR2 and HVEM) lack a death domain, and instead contain one or more TRAF (TNFR-associated factor) interacting motifs (TIMs), which trigger a variety of signal transduction pathways, including those for activation of nuclear factor κB (NF-κB), Jun N-terminal kinase (JNK), p38, extracellular signal-related kinase (ERK) and phosphoinositide 3-kinase (PI3K). Members of the third group (e.g., TNFR3, TNFR4, etc.) lack intracellular signaling motifs, and act as decoy receptors.

The natural ligands of the TNFRs are a family of cytokines whose prototype is TNF, and include lymphotoxin (LT)α, LTβ, and LIGHT. They are biologically active as trimers: their binding to the receptors causes the trimerization of the intracellular domains, which, in turn, interact with high affinity with trimeric cellular adaptors (e.g., TRAFs). Each cytokine interacts with more than one receptor.

HVEM

HVEM was first identified as a HSV receptor and was classified as a novel member of the TNFR family based on structural motifs (Montgomery *et al.*, 1996). The cytoplasmic tail interacts with several members of the TRAF family, leading to the activation of targets like NF-κB, Jun N-terminal kinase, and AP-1, and the consequent induction of T cell activation, proliferation, cytokine release, and expression of cell surface activation markers (Harrop *et al.*, 1998). Its ligands are $LT\alpha_3$ and LIGHT (Mauri *et al.*, 1998). LIGHT–HVEM interactions contribute to the cytotoxic T-lymphocyte-mediated immune response.

The main properties of HVEM as an HSV receptor are as follows.

(i) In early studies HVEM was found to be expressed mainly in cells of the immune system, and in a number of non-hematopoietic tissues and organs. Expression was not observed in brain or skeletal muscle (Montgomery *et al.*, 1996; Kwon *et al.*, 1997).

(ii) HVEM serves as receptor for HSV-1 and HSV-2, as a weak receptor for the U10 HSV mutant (L25P substitution), but not for HSV *rid*-1 and *rid*-2 unrestricted mutants (Connolly *et al.*, 2003; Yoon *et al.*, 2003).

(iii) HVEM binds wild-type gD. The affinity of the binding is of the same order of magnitude as that of nectin1/gD binding, and the interaction requires the same region of gD, i.e., the first 250 residues, or longer (Willis *et al.*, 1998). gD and LIGHT compete with each other for the binding to HVEM; accordingly, LIGHT interferes with HSV entry in HVEM-expressing cells (Mauri *et al.*, 1998).

(iv) The gD contact site on HVEM involves CRD1 and 2, with the majority of contacts lying in CRD1. Residues 35–37 form the intermolecular antiparallel β-sheet with gD (Carfi *et al.*, 2001; Connolly *et al.*, 2003). A systematic structure-based mutagenesis approach revealed that 17 residues in CRD1 and 4 in CRD2 are directly involved in the HVEM-gD interface. Some mutations completely abolish the HVEM binding to gD and its function as an HSV-1 receptor (Connolly *et al.*, 2002).

Modified heparan sulphate

3-O-sulphated heparan sulphate represents the third identified HSV receptor, structurally unrelated to nectins and HVEM (Shukla *et al.*, 1999). It serves as receptor for HSV-1, but not for unrestricted *rid* mutants or HSV-2. Thus, HSV-1 can use heparan sulphate GAGs not only for the initial step of attachment to the target cell, but it also recognizes some specifically modified sites on heparan sulphate (Shukla and Spear, 2001). The physical interaction with gD is in the range of 10^{-6} M, as measured by affinity co-electophoresis, and was not detectable by ELISA.

The sites on heparan sulphate recognized by gD are generated by heparan sulphate D-glucosaminyl 3-O-sulfotransferases (3-OSTs). 3-O-sulphates are rare substitutions in heparan sulphate, generated by at least six 3-OSTs isoforms identified in humans and mice, 3-OST-3_A and 3-OST-3_B, 3-OST-2, 3-OST-4 and 3-OST-5 (Shukla and

Spear, 2001). Of note, 3-OST-3s are not receptors *per se,* consistent with the finding that gD does not bind to the purified enzymes; rather, they catalyze the substitutions, which, in turn, generate the receptors and render CHO cells susceptible to HSV.

The relevance of this potential receptor to HSV infection in human cell lines and in humans remains to be ascertained. In terms of distribution, the 3-OST-3s are broadly expressed isoforms in cells of different origins and tissues; 3-OST-2 and 3-OST-4 expression is mainly in the brain. 3-OST-5 expression is limited to the skeletal muscle.

Receptor preference and usage

The availability of multiple alternative receptors raises a number of questions, such as: What receptor is preferred in cells that coexpress both molecules? Do both serve as *bona fide* receptors in humans? Are they differentially distributed, or employed in different tissues? Limited information is available on these topics.

Two parameters that may guide receptor preference in cell cultures are the affinity of the binding and the cell surface density of the receptors. Neither appears to be relevant in the case of gD, since gD affinity to nectin1 and to HVEM is of the same order of magnitude (Krummenacher *et al.*, 1998). Moreover, cells that appear to be nectin1-negative by fluorescent antibody cell sorting (e.g., EA-1 cells) can be infected and entry is inhibited by antibody to nectin1 (our unpublished result), implying that low density receptors suffice to mediate entry. The same phenomenon is observed with HVEM (Krummenacher *et al.*, 2004).

As far as infection of animal and human tissues is concerned, nectin1 is expressed in nearly every neuron of adult mice, in rat sensory neurons, in some synapses, and serves as the primary receptor for HSV-1 infection in sensory neurons (Haarr *et al.*, 2001; Mata *et al.*, 2001; Richart *et al.*, 2003). Nectin1 is also expressed in the epithelium of the human and murine vagina where it mediates HSV-1 and -2 entry in the genital mucosa of female hosts (Linehan *et al.*, 2004). Nectin1-α is detected at the cell–cell adherens junctions in human skin (Matsushima *et al.*, 2003). Cumulatively, these data are compatible with a major role of nectin1 in the infection of sensory neurons and mucoepithelia in vivo. The tissue distribution of HVEM suggests that it serves as the principal receptor for HSV in activated T lymphocytes, or in lymphoid organs (spleen and thymus), in liver and lung. However, these organs are not target of HSV in the natural history of infection, except in the rare cases of disseminated infection. Recent data show that several clinical/primary isolates can use both receptors, as laboratory strains do (Krummenacher *et al.*, 2004). The fact that viruses of different origin retain the ability to use both receptors suggests that this is a requirement for successful infection and spread in the host (Krummenacher *et al.*, 2004). The interaction with HVEM in particular may have a role in immune evasion (La *et al.*, 2002).

For other viruses, formal proof that a given receptor plays a critical role in humans has rested on correlations between genetic defects in the locus encoding the receptor and a diminished susceptibility to viral infection. A rare truncation of the nectin1 gene has been identified. The phenotypes associated with loss of both alleles include cleft lip/palate, hidrotic ectodermal dysplasia (CLPED1), hair abnormalities, developmental defects of the hands and, in some cases, mental retardation. Studies on the serostatus of these patients for evidence of HSV infection have not been published. A few studies investigated nectin1, nectin2 and HVEM gene polymorphisms and correlations with HSV infection. At present, no relatively common polymorphism has been found to correlate with HSV serostatus or symptoms (Patera *et al.*, 2002; Struyf *et al.*, 2002; Krummenacher *et al.*, 2004). Wild-type HSV can infect CLPED1 fibroblasts through HVEM in vitro, indicating that the ability to exploit redundant receptors may be favorable to the virus in vivo as well (Krummenacher *et al.*, 2004).

The animal orthologues of nectin and HVEM

HSV infects numerous mammalian species, some of which – mice, rabbits and primates – are extensively employed as animal models. The question arises whether they are faithful models in terms of receptor usage.

Porcine and bovine alphaherpesviruses promiscuously use human nectin1, implying that animal orthologs of nectins serve as receptors of these viruses in their hosts (Cocchi *et al.*, 1998b; Geraghty *et al.*, 1998; Warner *et al.*, 1998). Indeed, nectins are conserved among mammals, and nectin1 orthologs have been found in cells derived from mice, hamsters, pigs, cows and monkeys, and nectin2 has a mouse ortholog, suggesting a conserved function in the evolution of these proteins (Milne *et al.*, 2001). A HVEM ortholog is expressed in mice (Yoon *et al.*, 2003). In turn, the murine orthologs of nectin1, nectin2, HVEM, and the porcine and bovine hortologs of human nectin1 can serve as species non-specific HSV receptors when transfected into receptor-negative cells (Menotti *et al.*, 2000; Shukla *et al.*, 2000; Menotti *et al.*, 2001; Milne *et al.*, 2001; Menotti *et al.*, 2002a). The affinity of these potential receptors for gD ranges from as high as that of human nectin1, to very low or undetectable. Because the animal orthologs of nectin, and perhaps also of HVEM serve as receptors of HSV in mice and other animal species, these systems appear to relatively faithfully model HSV receptor usage in humans.

Site of HSV entry into the cell

It has been a long held paradigm that HSV enters cells by fusion at the plasma membrane. Recent evidence indicates that in some cells entry is through an endocytic pathway, and that both the cell type, and the structural features of the receptors are determinants that control the site of entry. Specifically, in cells like HeLa and CHO expressing nectin1 or HVEM, entry is inhibited by drugs that modify the pH of the endosomal compartment (low pH-sensitive entry) (Nicola *et al.*, 2003). However, when nectin1 or HVEM are expressed in J cells, they mediate entry at the plasma membrane. Furthermore, when nectin1 is retargeted to endosomes, by means of a chimeric nectin1-EGFR (epidermal growth factor receptor) chimera, or is sorted to lipid rafts, by means of a nectin1-glycosylphosphatidylinositol anchor chimera, the pathway of entry into J cells becomes endocytic (Gianni *et al.*, 2004). Of note, when HSV infects cells carrying wt-nectin1, neither nectin nor gD localize at lipid rafts, but gB does (Bender *et al.*, 2003). All in all, HSV fusion glycoproteins are well suited to perform two quite different pathways of entry.

Role of gD-receptor interaction in triggering fusion

Crucial to our understanding of how HSV enters cells is the comprehension of how gD binding to its receptor triggers fusion. A hint has come from the unexpected observation that the soluble gD ectodomain is both necessary and sufficient to rescue the infectivity of the non infectious gDnull HSV mutant (Cocchi *et al.*, 2004). Entry mediated by soluble gD requires not only the N-terminus, carrying the receptor-binding sites, but also the C-terminus carrying the pro-fusion domain, required to trigger fusion but not for receptor binding. These findings, together with the observation that a glycosylphosphatidylinositol-anchored form of gD, or substitution of the transmembrane and cytoplasmic tail with heterologous regions leave gD function unaltered (Browne *et al.*, 2003) demonstrate that the transmembrane region and cytoplasmic tail do not play any demonstrable function, except to ensure that gD is delivered to the gD receptor along with the virion, and argue that the role of gD in HSV entry is to signal receptor-recognition to the downstream glycoproteins and to trigger fusion (Cocchi *et al.*, 2004).

Biochemical and structural studies indicate that the receptor-mediated activation of gD takes the form of a conformational change (Cocchi *et al.*, 2004; Fusco *et al.*, 2005; Krummenacher *et al.*, 2005). In the unliganded state the virion gD adopts a conformation in which the flexible C terminus folds back, wraps the N-terminus and masks the receptor binding sites. At receptor binding, the C-terminus is displaced from its binding site on the N-terminus, the receptor binding sites are unmasked and become occupied by the receptor. The binary complex made of receptor plus gD with the displaced C-terminus must create a surface suitable for gB and gH-gL recruitment.

Execution of membrane fusion and its control

gB, gH, gL are essential for entry of all herpesviruses, since they are conserved among all human herpesviruses, with the highest extent of sequence conservation seen in gB. Heterodimer formation between gH and gL is also a conserved feature amongst herpesviruses. Altogether, gB, gH and gL appear to be the executors of fusion and constitute the conserved fusion machinery across the herpesvirus family.

Critical properties of gH and gB have been elucidated recently, and provide an intriguing scenario. On one hand, molecular and biochemical analyses of gH highlighted properties typical of class 1 fusion glycoproteins. Because the gH structure has not yet been solved, these properties wait for confirmation at the structural level. On the other hand, the crystal structure of gB has been solved, it exhibits a remarkable similarity to that of vesicular stomatitis G protein, and to viral fusion glycoproteins in general. Biochemical and mutational confirmation are still to be provided. At present, a most likely scenario is that both gB and gH·gL are fusion executors. How the two glycoproteins cooperate to execute fusion, and why two, and not one, fusion executors are required in the herpesviridae family is unclear. It is worthwhile to note that entry by fusion at plasma membrane, and entry by fusion in endocytic vesicles require all four glycoproteins (gD, gB, gH and gL) (Nicola *et al.*, 2003; Nicola and Straus, 2004). These requirements rule out the possibility that gB serves as fusion executor in one cellular compartment, and gH·gL serves as fusion executor in another cell compartment.

The glycoproteins that execute fusion

gH –gL

gH is a type-1 virion glycoprotein encoded by the UL22 gene (Gompels and Minson, 1986). Soon after its discovery, it was recognized as an essential glycoprotein for virion infectivity, as its deletion produced non infectious progeny and abolished cell–cell fusion (Forrester *et al.*, 1992). Neutralizing antibodies to gH block virus entry but permit attachment, indicating a role at a post-attachment step (Fuller *et al.*, 1989). gH appears to contain elements associated with fusion of membranes, i.e. a hydrophobic α-helix 1 (residues 377-397) with properties typical of a fusion peptide and two heptad repeats with propensity to form a coiled coil. α-Helix 1 is positionally conserved in all the

gH orthologs across the herpesviridae family; in HSV-2 it is located in a loop made of two cysteins. α-Helix 1 is able to interact with biological membranes, can convert a soluble glycoprotein (gD amino acid residues 1-260) into a membrane-bound glycoprotein, and can be functionally replaced by fusion peptides derived from glycoproteins of other, unrelated viruses (Gianni *et al.*, 2005a). A peptide with the sequence of α-helix 1 induces fusion of liposomes and exhibits a strong flexibility documented as ability to adopt an α-helical conformation (Galdiero, S. *et al.*, 2006; Gianni *et al.*, 2006a). These properties strongly argue in favor of α-helix 1 as a candidate fusion peptide loop. Two heptad repeats, capable to form coiled coils and to interact with each other, form a structure of increased α-helical content and are potentially suitable to form a six-helix bundle (Gianni *et al.*, 2005b; Galdiero, S. *et al.*, 2006; Gianni *et al.*, 2006b). Additional elements in gH are a second predicted α-helical domain of lower hydrophobicity than the candidate fusion peptide (aa 513-531), and a pre-transmembrane sequence (aa 626-644) with predicted propensity to partition at membrane interface (Galdiero, S. *et al.*, 2006; Gianni *et al.*, 2006a).

Synthetic peptides corresponding to the heptad repeats inhibit virus infection if present at the time of virus entry into the cell (Gianni *et al.*, 2005b; Galdiero, S. *et al.*, 2006; Gianni *et al.*, 2006b). The presence of coiled coil motifs predicts that gH must undergo profound conformational changes at fusion. Because fusion peptides and coiled coil heptad repeats represent characteristic functional domains in type 1 viral fusion glycoproteins, gH is a candidate fusion executor in HSV.

It remains to be determined whether gH interacts with cellular receptors. The interaction with an integrin is not critical given that mutagenesis of a RGD motif did not reduce virus entry and cell fusion (Galdiero, M. *et al.*, 1997). It is of interest that the transmembrane and C-terminal tail regions of gH can not be exchanged with those of heterologous proteins, in contrast with what happens with gD (Harman *et al.*, 2002; Jones and Geraghty, 2004).

A *ts* mutant, tsQ26, exhibited a phenotype characterized by the production of non-infectious extracellular virions, along with the intracellular retention of gH and of infectious virions (Desai *et al.*, 1988). This phenotype suggested a peculiar mechanism of intracellular retention of gH. A clue to understanding the intracellular trafficking of gH came from the observations that, when expressed from a transgene, gH had a M_r lower than that of mature gH, was not transported to the cell surface, and was retained in the ER unless the cells were superinfected (Gompels and Minson, 1986; Foà-Tomasi *et al.*, 1991; Roberts *et al.*, 1991). The gene product required for gH trafficking and maturation, identified by Johnson and coworkers, is gL (Hutchinson *et al.*, 1992a); gL is required for proper folding and traffiking of gH in all human Herpesviruses (Kaye *et al.*, 1992; Liu *et al.*, 1993).

gL is a soluble glycoprotein encoded by UL1 gene; its presence in the virion envelope is ensured by complex formation with gH (Hutchinson *et al.*, 1992a). In accordance with gH attributes, an HSV mutant unable to express gL could not enter cells, and its particles lacked glycoprotein H (Roop *et al.*, 1993). Both gH and gL are required for fusion in the cell–cell fusion assay (Turner *et al.*, 1998). The first 323 amino acids of gH and the first 161 amino acids of gL can form a stable secreted hetero-oligomer, while the first 648 amino acids of gH are required for reactivity to conformation-dependent antibodies, indicative of correct conformation and oligomerization (Peng *et al.*, 1998). gL is a locus of a *syn* mutation, confirming a role of the gH-gL hetero-oligomer in HSV fusion. The exact role of gL in fusion remains to be elucidated. Because its binding site on gH maps both upstream and downstream of the hydrophobic α-helix, it has been proposed that its role may be to shield the gH hydrophobic sequence, and thus to enable gH water solubility and solvent interface (Gianni *et al.*, 2005a).

gB

gB plays two opposite roles in fusion, i.e. it participates in fusion execution, and it exerts anti-fusion activity. The two functions are physically separated and reside in the ectodomain and the cytoplasmic tail, respectively. gB is a type-1 virion glycoprotein encoded by the UL27 gene (Bzik *et al.*, 1984; Pellett *et al.*, 1985). Its crystal structure reveals a trimer with a coiled coil core. Remarkably, its structure resembles closely that of vesicular stomatitis virus G protein (Heldwein *et al.*, 2006; Roche *et al.*, 2006). Despite the facts that a canonical fusion peptide has not been detected by biochemical, molecular or structural analyses, and that the region homologous to the fusion peptide loop in vesicular stomatitis virus G protein appears to be suboptimal for membrane insertion, the structural similarity between gB and vesicular stomatitis virus G protein strongly relates gB to viral fusion glycoproteins. It has been proposed that the two glycoproteins may represent a novel class of fusion glycoproteins (Heldwein *et al.*, 2006; Roche *et al.*, 2006).

From a structural point of view, gB is a trimeric spike. Each of the three protomers (residues 103–730) appears to be composed of five distinct domains (named I-V), displaying multiple contact sites (Heldwein *et al.*, 2006). Domain I, the "base", is a continous chain with a fold typical of pleckstrin homology domains. Domain II, the "middle", is made of two discontinuous segments, forming a structure

reminiscent of a pleckstrin homology superfold. Domain III, the "core", comprises three discontinuous segments: its prominent feature is a 44-residue α-helix that forms the central coiled coil with its trimeric counterparts. Domain IV, the "crown", adopts a novel structure, and is fully exposed on top of the trimeric spike. Domain V, the "arm", is a long extension spanning the full length of the protomer. Of note, its residues do not contact residues of the same protomer, but instead accommodate into the groove formed by the "cores" of the other two protomers.

The role of gB in virion infectivity and cell–cell fusion is inferred by numerous lines of evidence, including (i) the phenotype of a gB deletion mutant virus which produces non-infectious particles, (ii) the neutralizing activity of antibodies to gB, (iii) gB as a genetic locus of syncytial mutations, and (iv) the requirement for gB in the cell-cell fusion assay (Manservigi *et al.*, 1977; Cai, W. Z. *et al.*, 1987; Turner *et al.*, 1998). Functional domains in the ectodomain were identified by means of two sets of mutations: temperature sensitive mutations for viral growth, and resistance to antibodies with potent neutralizing activity. The first ones, exemplified by the mutants tsB5 and tsJ12, reside in the gB ectodomain, confer a temperature-sensitive phenotype, and affect the rate of virus entry (Bzik *et al.*, 1984). Likely, these mutations affect the gB domain involved in execution of fusion. Following the determination of gB crystal structure, it was recognized that the epitopes of potent neutralizing antibodies, either centered around single amino acid residues or formed by continuous regions, reside on the trimer surface, on the lateral faces of the spike or on the tip of the crown (Pellett *et al.*, 1985; Kousoulas *et al.*, 1988; Highlander *et al.*, 1989; Pereira *et al.*, 1989; Qadri *et al.*, 1991; Heldwein *et al.*, 2006).

The quartet of gD, gB, gH and gL assemble into a complex at virus entry

The nature of the interactions between the complex formed by gD plus its receptor and the executors of fusion is critical to understand the mechanisms by which HSV (and by extension all other herpesviruses) enter cells.

The quartet of glycoproteins essential for HSV entry and fusion (gD, gB, gH and gL) assemble into a complex at virus entry and in infected cells. Complex assembly strictly requires one of the gD receptors, either nectin1 or HVEM. The same complex is assembled also in cells transfected with the quartet, implying that no additional viral protein other than those that participate in the complex itself is required. Because the complex is assembled at virus entry and in transfected cells committed to form polykaryocytes, and fails to be assembled in the absence of either a receptor to gD or of gD, complex assembly appears to be a critical step in the process of virus entry and fusion.

The proteins that negatively control fusion

Cells infected with wt-virus do not form syncytia, despite the fact that they express the fusion glycoproteins at their surface. Syncytia are only formed when the virus carries one of the syncytial (*syn*) mutations, which map to genes encoding gB, gL, gK, UL24, or UL20. By contrast, cells expressing the quartet of gB, gD, gH and gL readily form syncytia (Turner *et al.*, 1998). The paradox may be explained by assuming that the wt-alleles of proteins that are target of *syn* mutations exert a negative control on fusion. This has, in fact, been verified for gB, gK, and UL20 (Fan *et al.*, 2002; Avitabile *et al.*, 2003; Avitabile *et al.*, 2004). As outlined below, HSV has evolved at least two mechanisms by which it blocks fusion. One is exerted through downmodulation of gB cell surface expression, the other is exerted through the concerted action of gK and UL20p. Still other proteins (UL24 and UL45) are likely to exert anti-fusion activity. The evolution of functional redundancy implies that uncontrolled fusion is inimical to HSV-1 replication and spread in nature, and therefore the virus needs to exert a tight control on it.

gB

The anti-fusion activity of gB is located in the cytoplasmic tail, which carries at least two physically distinct functional domains: the *syn* mutation and the endocytosis motifs. Each of them, separately, appears to reduce fusion. Structurally, the cytoplasmic tail carries two predicted α-helices. The *syn* mutations are located immediately downstream of the most N-terminal α-helix. Embedded in the region of the C-terminal α-helix is one, and possibly two functional endocytosis motifs (YTQV889–892 and LL871 (Fan *et al.*, 2002; Avitabile *et al.*, 2004; Beitia Ortiz de Zarate *et al.*, 2004). Deletion of the membrane-proximal α-helix abrogates virus infectivity, implying that this region is critical. Its role, and the molecular mechanism of the *syn3* mutation remain to be elucidated. The membrane-distal α-helix is also implicated in the negative control of fusion, since its deletion increases fusion in the cell fusion assay, and confers a syncytial phenotype upon virus-infected cells (Foster, *et al.*, 2001a; Avitabile *et al.*, 2004). Its antifusion activity is mainly exerted through endocytosis, which acts to decrease the steady state amounts of gB from the cell surface, such that gB becomes a limiting factor in fusion. Of note, the gB-decorated endocytosis vesicles-vacuoles represent the hallmark of gB localization in infected cells.

gK

gK is a polytopic glycoprotein encoded by the UL53 gene, whose topology is still debated (Hutchinson *et al.*, 1992b; Foster, *et al.*, 2001b). It carries an N-terminal extracellular domain, and two or three TM regions connected by loops (Foster, *et al.*, 2003b). Its hydrophobicity, poor immunogenicity and overall problems in its detection have made this glycoprotein a difficult one to study. In infected cells, gK localizes mainly to the Golgi apparatus. One controversial aspect is whether it localizes to the plasma membranes and to virions. When expressed from a transgene, gK is primarily located is at the ER (Hutchinson *et al.*, 1992b; Avitabile *et al.*, 2003; Foster, *et al.*, 2003a). When coexpressed with UL20, both proteins localize to the Golgi apparatus (Avitabile *et al.*, 2004).

gK exerts anti-fusion activity in the cell–cell fusion assay (Avitabile *et al.*, 2003). Mutant viruses carrying a partial or a complete deletion in the gK gene have two major phenotypes (Hutchinson and Johnson, 1995; Foster, and Kousoulas, 1999). First, they form syncytia, arguing that the anti-fusion activity is exerted also in the context of infected cells. Second, they are defective in virus egress, arguing that the anti-fusion activity of gK is exerted not only at the plasma membrane, but also in the membranes of the exocytic compartment. This would provide an explanation as to why these membranes are heavily decorated with fusion glycoproteins, yet do not fuse one with the other. According to this model, the gK role in virion egress may be exerted by maintaining a functional exocytic pathway. It should be stressed that, if indeed gK is also a virion constituent, then, at virus entry into the cell, the trigger to fusion must simultaneously relieve the block to fusion exerted by gK (Avitabile *et al.*, 2004).

UL20

UL20p is a polytopic unglycosylated protein with several analogies to gK. Its hydrophobicity and scarce immunogenicity have hampered its characterization. UL20p is predicted to carry 4 transmembrane segments (McGeoch *et al.*, 1988; Melancon *et al.*, 2004). In the infected cells UL20p localizes at the Golgi apparatus and the nuclear membranes, and is not detectable at the plasma membrane. It has not been detected in virions. When expressed from a transgene, UL20p predominant localization is at the ER (Avitabile *et al.*, 1994; Ward *et al.*, 1994). It relocalizes to the Golgi apparatus, when coexpressed with gK (Foster, *et al.*, 2003b; Avitabile *et al.*, 2004).

Two mutant viruses deleted in UL20 gene have been constructed, both of which are highly defective in secretion of virions to the extracellular space (Baines *et al.*, 1991; Foster, *et al.*, 2004). The first deletion virus was subsequently reported to carry an in-frame fusion between UL20.5 (not known at the time the deletion virus was constructed) and the C-terminus of UL20 gene, and was characterized by syncytia formation and by the entrapping of virions in the perinuclear space (i.e., the space between the inner and outer nuclear membranes) – a phenotype particularly evident in cells whose Golgi apparatus became fragmented following infection (Baines *et al.*, 1991). This phenotype can be interpreted as indication that the UL20p exerts a negative control on fusion. Cells infected with the second deletion virus showed enveloped virions as well as unenveloped nucleocapsids accumulating in the cytoplasm, and occasionally virion envelopes containing multiple capsids within intracytoplasmic vacuoles. These phenotypes were also interpreted to mean that UL20p acts as an inhibitor of membrane fusion, and, interestingly, that UL20p may act to maintain a single nucleocapsid for each envelope and to prevent fusion of enveloped virions among themselves (Foster, *et al.*, 2004). The complexity of these phenotypes reflects both direct and indirect effects of UL20p, including the role of UL20p in the intracellular transport of gK and possibly of the fusion glycoproteins.

The possibility that UL20p exerts an anti-fusion activity was probed in the cell–cell fusion assay, which showed a block to fusion in cells coexpressing UL20p and gK, but not in cells expressing UL20 alone. The block was cell line dependent (Avitabile *et al.*, 2004). The similar behavior of gK and UL20p, their colocalization, their mutual ability to influence each other localization, and their concerted anti-fusion activity make it likely that the two proteins act in a complex, and that they share a common target.

Nucleocapsid transport to the nuclear pore

Virus entry culminates in the release of capsids and approximately twenty tegument proteins into the cytosol. The capsids and some of the tegument proteins, e.g., αTIF, travel to the nuclear pore. Since diffusion of molecules larger than 500 kDa is restricted in the cytoplasm, viruses and nucleocapsids require a transport system. This is particularly true for neurotropic viruses that travel long distances in the axon during retrograde or anterograde transport (Enquist *et al.*, 1998). It has been calculated that in the absence of an active transport mechanism, it would take a herpes virus capsid 231 years to diffuse 10 mm in the axonal cytoplasm (Sodeik, 2000).

Microtubules represent the cytoplasmic highways on which HSV is transported (reviewed in Döhner and Sodeik, 2005). At virus entry, capsids co-localize with microtubules, and their depolymerization reduces capsid transport to

the nucleus (Sodeik *et al.*, 1997; Mabit *et al.*, 2002). Microtubules are polar structures with fast growing plus-ends typically localized in the cell periphery, and less dynamic minus-ends that are usually anchored in close proximity of the nucleus at the microtubule organizing centre (MTOC). Molecular motors use ATP-driven conformational changes to transport cargo along microtubules. Transport to the plus-ends is catalyzed by kinesins and that to minus-end by cytoplasmic dynein and dynactin (Döhner and Sodeik, 2005). Dynein and dynactin mediate capsid transport to the cell centre, since incoming capsids colocalize with these motors (Sodeik *et al.*, 1997; Döhner *et al.*, 2002), and overexpression of dynamitin, a subunit of the dynactin complex, inhibits capsid transport (Döhner *et al.*, 2002). How capsids move further from MTOC to the nuclear pore complex is unclear.

Analysis of HSV-1 entry by digital time-lapse fluorescence microscopy showed that GFP-tagged capsids can move along microtubules both towards and away from the nucleus, with maximal speeds of 1.1 μm/s. The transport is saltatory and bidirectional, but in neuronal processes it shows a retrograde bias towards the cell body (Smith *et al.*, 2001). Efforts are underway to identify the virion proteins that may interact with kinesins as well as dynein or dynactin. Two candidates are UL34p, which, however, is absent from mature virions, and US11, which appears to bind the heavy chain of conventional kinesin (Diefenbach *et al.*, 2002). Once the capsids have reached the proximity of the nucleus, they seem to bind to filaments emanating from the nuclear pores (Batterson *et al.*, 1983; Sodeik *et al.*, 1997). This docking is believed to induce capsid destabilization, release of the viral DNA, and its translocation through the nuclear pore into the nucleoplasm (Ojala *et al.*, 2000). Temperature-sensitive mutants in the UL36 gene accumulate filled viral capsids at the nuclear pore complexes at the non-permissive temperature, suggesting that the large tegument protein VP1–3 is involved in uncoating of the viral genome (Batterson *et al.*, 1983; Ojala *et al.*, 2000).

VZV

There are several remarkable differences between VZV and HSV entry. Because the respective viral glycoproteins undoubtedly influence these differences, the VZV gene products will be briefly summarized.

Is VZV gE a substitute for functions of HSV gD?

All but one of the proteins that have been illustrated above for HSV have a counterpart in VZV. For those glycoproteins for which sufficient information is available, a substantial functional similarity is observed. The single most notable difference between VZV and HSV in terms of the glycoproteins is the absence of gD in the VZV genome. At the same time, VZV is well suited for cell-to-cell spread, which takes place by fusion of the infected cell with an adjacent uninfected cell, whereas HSV is better suited for virion-to-cell infection (at least in cultured cells). So, it is tempting to speculate that the absence of a VZV gD gene may contribute to these differences.

Despite the fact that gD plays such a pivotal role in HSV entry, gD is not conserved throughout the alphaherpesviruses. In the porcine herpesvirus PrV, gD is required for virion-to cell infectivity but not for cell-to-cell spread of the virus. Consistently, gD is not a requirement for the PrV cell-cell fusion assay, although its presence greatly enhances fusion efficiency. Assuming that common basic mechanisms are shared by all of the alphaherpesviruses and given that the triplet gH-gL-gB is conserved, the question then arises: which VZV glycoprotein substitutes for the functions encoded in HSV gD, i.e., receptor recognition and triggering of fusion. The two functions might well be distributed over different entities, but a trigger to fusion consequent to virion interaction with a receptor appears to be essential.

In VZV, four glycoproteins are known to be essential. They are gB, gH, gL and gE (Keller *et al.*, 1984; Montalvo and Grose, 1986; Forghani *et al.*, 1994; Duus *et al.*, 1995; Mallory *et al.*, 1997; Mo *et al.*, 2002). Of note, the HSV gE gene lies in the S component of the genome, proximal to gD; as stated above, VZV lacks the gD gene. Instrumental to our understanding of the role of gE are the results of VZV glycoprotein cell–cell fusion assays. In transfected cells, fusion is induced by coexpression of either gH-gL or of gB-gE (Duus *et al.*, 1995; Duus and Grose, 1996; Maresova *et al.*, 2001). With regard to genome stability, VZV is considered to be one of the more genetically stable herpesviruses. However, viral mutants carrying missense mutations in the gE ectodomain are being isolated from humans; one of them is more fusogenic in cell cultures and in the SCID-hu mice (Santos *et al.*, 1998, 2000). Cumulatively, both circumstantial and genetic evidence supports the possibility that VZV gE subsumes at least some of roles of HSV gD.

Endocytosis of the VZV glycoproteins gE, gB, gH, and the negative regulation of fusion

Three VZV glycoproteins carry functional tyrosine-based endocytosis motifs; they are gE, gB and gH.

The gE cytoplasmic tail has a YAGL sequence beginning with a tyrosine residue 582. As determined by mutagenesis studies, the tyrosine residue is part of a conserved YXXL endocytosis motif. The internalized gE trafficks to the trans-Golgi or is recycled to the cell surface. In addition, the C-tail also contains phosphorylation sites (Kenyon *et al.*, 2002). It has been suggested that serine/threonine and tyrosine phosphorylation of gE may serve as sorting signals for internalized receptors and that formation of a gE–gI complex facilitates gE endocytosis (Olson and Grose, 1997; Olson *et al.*, 1998).

VZV gB contains three predicted endocytosis motifs within its cytoplasmic domain: YMTL (aa 818–821), YSRV (aa 857–860), and LL (aa 841–842). Both tyrosine-based motifs mediate gB internalization, but only the YSRV motif is absolutely required for endocytosis. The YMTL motif functions in trafficking of internalized gB to its subsequent localization in the trans Golgi. The third potential endocytosis motif is a dileucine sequence, whose function is under study (Heineman and Hall, 2001). Of note, VZV gI, the partner of VZV gE, also contains a dileucine endocytosis motif in its C-tail (Olson and Grose, 1998).

Like VZV gE and gB, VZV gH contains a functional but previously unrecognized tyrosine based YNKI motif in its short cytoplasmic tail, which mediates clathrin-dependent and antibody-independent endocytosis. Alignment analysis of the VZV gH cytoplasmic tail with other herpesvirus gH homologues reveals two interesting features: (i) herpes simplex virus types 1 and 2 homologues lack an endocytosis motif while all other alphaherpesvirus gH homologues contain a potential motif, and (ii) the VZV gH C-tail is actually longer than predicted in the original sequence analysis and thus can provide the proper context for a functional endocytosis motif (Pasieka *et al.*, 2003). Surprisingly, the endocytosis-deficient VZV gH mutant plasmid effects greater cell-cell fusion than the wild-type gH plasmid. This result leads to the conclusion that VZV gH endocytosis represents a mechanism through which cell–cell fusion is negatively regulated, i.e., by modulating the amount of fusogenic gH on the cell surface (Pasieka *et al.*, 2004). In this respect, therefore, VZV gH shares a basic mechanism of negative regulation of fusion with HSV gB.

Cumulatively, this comparison of the VZV and HSV-1 systems is very instructive as it highlights that both viruses have evolved an essentially similar mechanism of control of fusion, based on endocytosis and consequent limitation of cell surface expression of the fusion executors themselves. A notable difference between the two viruses is that this type of control appears to be is exerted in HSV-1 mainly by gB and in VZV mainly by gH.

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8

Early events pre-initiation of alphaherpes viral gene expression

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The regulated transcription of the HSV IE (immediate–early, α) genes has been a model system for elucidating principles and mechanisms of combinatorial-differential regulation, basic RNAPII-directed transcription, and multiprotein assembly specificities. The regulation exemplifies viral mechanisms dedicated to the recruitment of cellular components into complex viral–host interactions that illustrate general parameters of protein–protein, DNA–protein, RNA transcription, and protein complex assembly. Continued studies hold promise of advancing the understanding of the complexities of biochemical interactions in gene expression as well as complex cellular response pathways. The regulation of the IE genes within specific contexts may also lead to the understanding of signals and pathways which modulate viral infection and determine the extent of lytic-latent infection. While HSV has been extensively studied and will represent the focus of this review, the regulatory domain of the VZV IE gene (IE62) contains similar elements and is regulated by similar mechanisms.

The HSV IE regulatory domains: multiple sites for differential regulation

The regulatory domains of the HSV IE genes have been the focus of numerous studies that have defined the sequence elements and their contributions to the basal and induced levels of transcription. These IE domains typically consist of a reiterated inducible enhancer core element (consensus: TAATGARAT) that is flanked by binding sites for members of the ets and kruppel transcription family (Fig. 8.1, left) (Roizman and Sears, 1996; Vogel and Kristie, 2001).

The primary focus has been on the regulated induction of the expression of the IE genes by the HSV IE transactivator (VP16, αTIF, ICP25) via the enhancer core element (Phillips and Luisi, 2000; Roizman and Sears, 1996; Roizman and Sears, 1996; Vogel and Kristie, 2001; Vogel *et al.*, 2001; Wysocka and Herr, 2003). This focus led to the identification of cellular proteins (Oct-1 and HCF-1) that are required for the stable enhancer complex assembly and the induction of the IE gene transcription (Kristie *et al.*, 1989, 1995; Kristie and Sharp, 1993; Roizman and Sears, 1996; Vogel and Kristie, 2001; Vogel *et al.*, 2001; Wilson *et al.*, 1993; Wysocka and Herr, 2003). Extensive characterization of these components illustrates the multiple levels of complex regulatory interactions inherent in this process.

The assembly of the HSV IE enhancer core complex

The assembly of the HSV IE enhancer core complex illustrates the basic elements of the specificities governing the regulatory process. The stages of the assembly are modeled upon in vitro studies of protein–DNA recognition, protein-protein interactions, selective recognition, affinities, and cooperative interactions. As shown in Fig. 8.1 (Right), the cellular Oct-1 POU domain protein recognizes the divergent octamer element in the IE core and provides a nucleation point for the association of the heterodimeric protein complex consisting of VP16 and HCF-1. Specificity for the HSV IE element is determined by selective recognition of the Oct-1 POU-homeobox and the enhancer core DNA sequences by VP16 while the complex is stabilized by HCF-1 interactions. The full enhancer domain complex contains additional cellular transcription factors such as GABP and Sp1. The activation potential is dependent upon the core complex, likely through additional protein–protein interactions. The complexity of the regulatory complex provides for multiple levels of regulation dictated by various protein combinations, interactions, and the specific regulation of the individual components. Therefore, each component

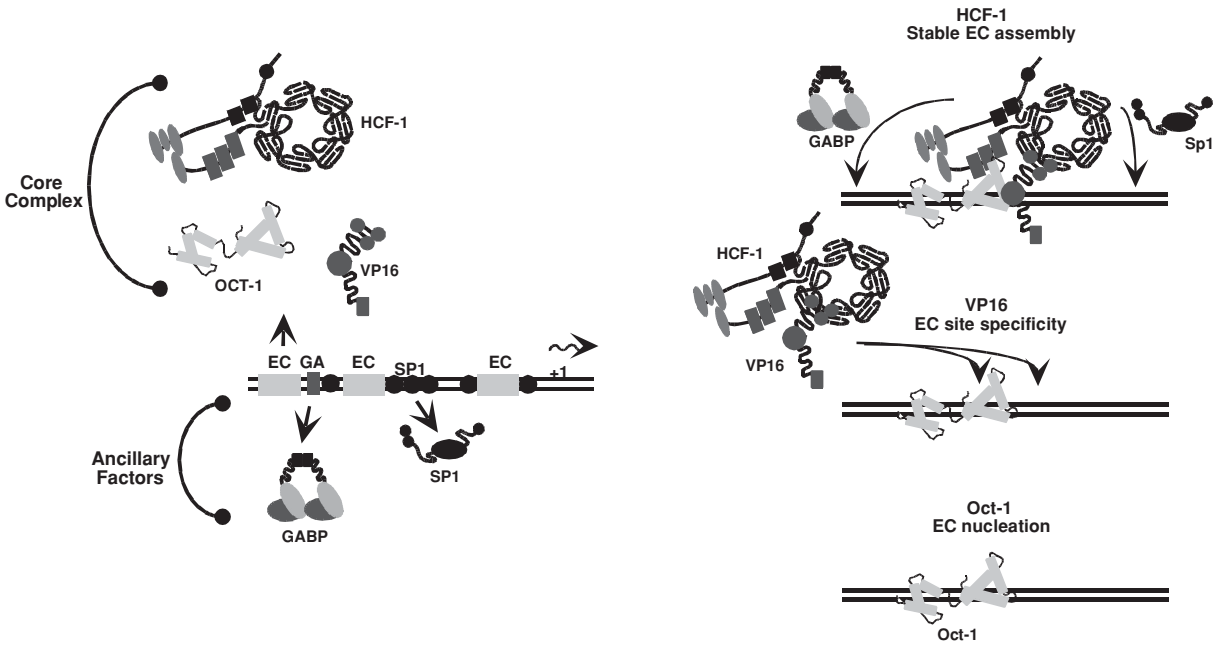

Fig. 8.1. The HSV IE enhancer domain elements, components, and assembly process. (Left) Representation of a typical HSV IE enhancer-promoter domain containing the reiterated enhancer core (EC), GABP (GA), and Sp1 (Sp1) binding sites. The enhancer core components (HCF-1, Oct-1, VP16) and ancillary transcription factors (GABP, Sp1) are represented. (Right) Specificities and interactions in the assembly of the multiprotein enhancer core complex. Oct-1 binds to the octamer element in the 5′ end of the ATGCTAATGARAT enhancer core and nucleates the association of the heteromeric HCF-1/VP16 protein complex. VP16 cooperatively interacts with the Oct-1 POU-homeobox and recognized the 3′ sequences of the core element. The stable enhancer core complex may recruit or interact with the additional factors to form the fully assembled enhancer complex.

will be described with respect to its defined structure, function, interactions, and regulatory mechanisms.

Oct-1

Oct-1 is a representative of the POU family of proteins (Pit, Oct, unc86) containing bipartite DNA binding domains (POU-specific box, POUs and POU-homeobox, POUh) consisting of helix-turn-helix structures that cooperate to recognize the consensus element (ATGCAAAT) (Clerc *et al.*, 1988; Phillips and Luisi, 2000; Sturm *et al.*, 1988). The structure and DNA contacts of the POU-specific box are analogous to the λ repressor in which all four helices make significant network DNA backbone contacts while DNA recognition by the POU-homeobox is characteristic of homeodomains in which helix 3 lies in the major groove and provides sequence recognition. The two DNA binding units recognize bases (POUs, ATGC; POUh, AAAT) in the major groove on opposite sides of the helix and are separated by a flexible and disordered linker region (Fig. 8.2-Oct-1) (Klemm *et al.*, 1994).

In the case of the HSV IE domains, Oct-1 nucleates the enhancer core assembly by binding the divergent octamer sequences of the core consensus (ATGCTAAT). Selective recognition of the Oct-1 POU-homeobox by VP16 was elucidated by comparative analysis of Oct-1 and the highly related Oct-2 protein. While only seven amino acids differ between the exposed homeodomain surfaces of these two proteins, VP16 selectively interacts with Oct-1(Lai *et al.*, 1992; Pomerantz *et al.*, 1992). As shown (Fig. 8.2-Oct-1), four amino acids (K18, S19, E22, E30) in helixes 1 and 2 are required for high affinity recognition by VP16. Strikingly, only the single position 22 (Oct-1, E22) determines the specificity that accounts for the affinity difference in the recognition of Oct-1 over Oct-2 by VP16. This selective recognition by VP16 elucidated a principle of homeobox protein interaction and specificity that has been reflected in multiple Oct-1 interactions in differential positive, negative, and inducible regulatory events. Studies addressing these contextual interactions elucidate the functions of the protein and illustrate the various diverse mechanisms used to regulate these processes:

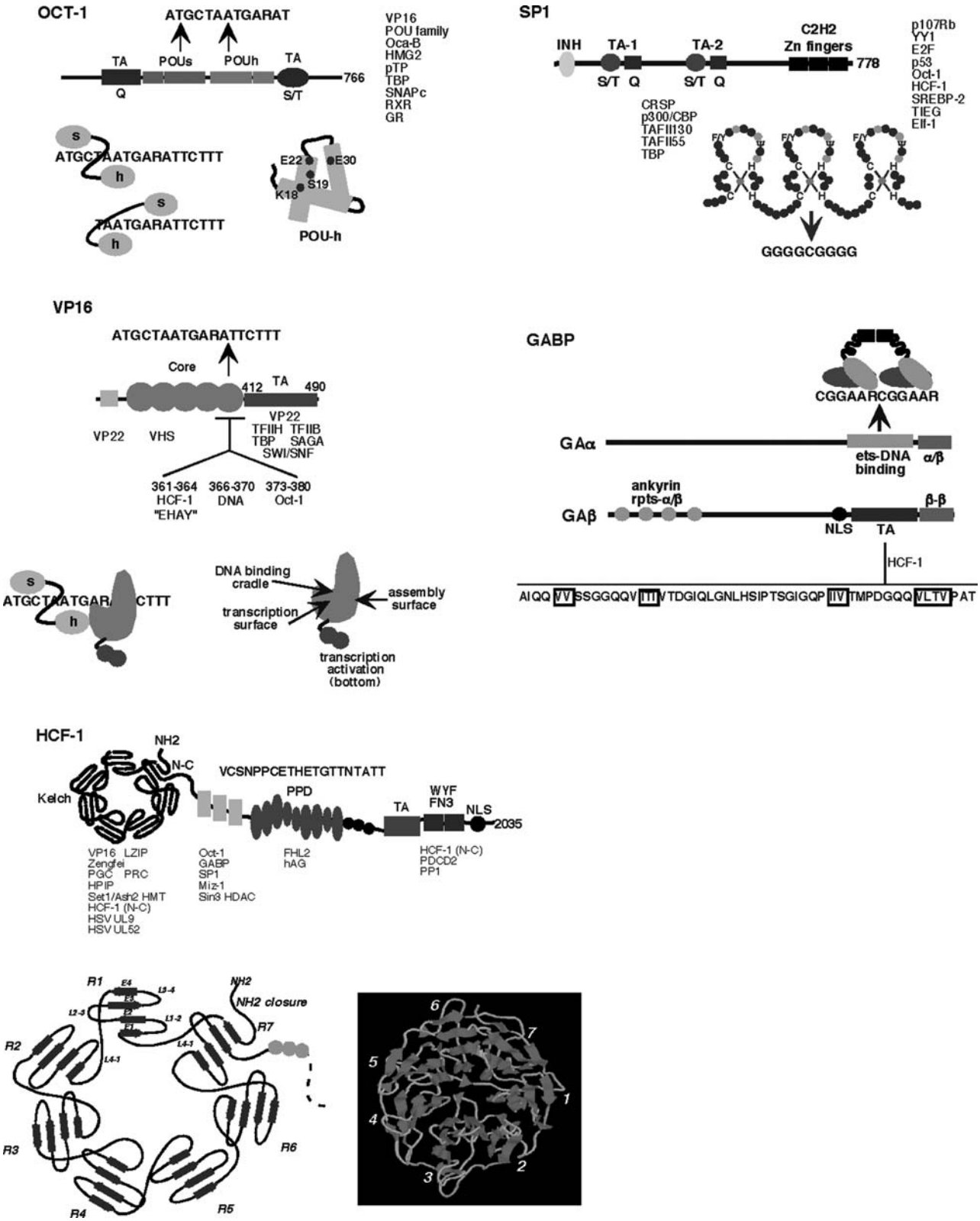

Fig. 8.2. The protein components of the HSV IE enhancer complex.
Oct-1: The transactivation (TA-Q and TA-S/T) and POU (POUs and POUh) domains are shown. The POU-specific box recognizes ATGC while the POU-homeobox recognizes TAAT within the enhancer core element (ATGCTAATGARAT). Proteins that bind to the Oct-1 POU domain are listed. The inherent flexibility of the POU domain and the potential orientations of the POUs box in recognition of the core element are depicted. In the schematic representation of the Oct-1 POU-homeobox, the residues which are important for the recognition by VP16 are indicated. (*cont.*)

(i) The stimulation of transcription preinitiation complex formation via the interaction of the Oct-1 POU domain with TBP in a non-DNA dependent manner has suggested that the interaction enhances TBP-TATA box binding by alleviating TBP autorepression, thus resulting in synergistic activation of octamer containing promoters (Mittal *et al.*, 1999; Zwilling *et al.*, 1994). Similarly, snRNA transcription is regulated via Oct-1/SNAPc interaction in which SNAPc is recruited to the snRNA proximal element via interactions that relieve SNAPc autorepression (Mittal *et al.*, 1999). The interaction of Oct-1 POU with SNAPc utilizes the same POU interaction surface as is bound by specific Oct-1 coactivators illustrating that the DNA site context is a determining factor in the selection of cofactors and coactivators (Hovde *et al.*, 2002).

(ii) Oct-1 stimulates transcription from TATA-less promoters via recruitment of TFIIB, functioning in lieu of TBP to orient the basal factor for RNAPII positioning (Phillips and Luisi, 2000).

(iii) Numerous synergistic interactions with other transcription factors also determine the roles of Oct-1 in positive, negative, and inducible regulation of gene expression. This regulation may be DNA dependent, promoter context dependent, or via DNA-independent protein-protein interactions. At the mouse mammary tumor virus (MMTV) promoter, interaction of the glucocorticoid receptor with the Oct-1 POU domain mediates glucocorticoid induction. Similarly, interaction with the androgen receptor promotes SRC-1 coactivator recruitment and transcription enhancement (Phillips and Luisi, 2000). In contrast, Oct-1 POU-homeobox interaction with the zinc finger domain of RXR disrupts the thyroid hormone receptor TR/RXR heterodimer, leading to repression of TR-dependent transcription (Gonzalez and Robins, 2001).

At the cyclin D1 promoter, interaction of CREB with the Oct-1 POU domain occurs independently of an octamer element and results in CBP coactivator recruitment in the absence of the normally responsive mechanism that depends upon CREB phosphorylation (Boulon *et al.*, 2002). Induction of GADD45 following UV-induced DNA damage is a second example of Oct-1 mediated regulation that is distinct from the defined primary p53 dependent regulatory mechanism (Jin *et al.*, 2001).

(iv) Another level of regulation is exemplified by Oct-1 interactions with cell-type or process-specific coactivators (Phillips and Luisi, 2000; Wysocka and Herr, 2003). The stimulation of immunoglobulin gene transcription by Oct-1 is mediated by binding of the B-cell specific coactivator OCA-B that interacts in a hydrophobic pocket with both subdomains of the Oct-1-POU domain. This interaction requires the Oct-1-DNA assembly, preferentially interacting with Oct-1 bound to the consensus ATGCAAAT vs. the HSV ATGCTAAT. The selection of site-specific Oct-1-DNA complexes by OCA-B is determined by binding a different surface of the Oct-1 POUs/POUh subdomains than VP16 that is positioned to the center of the consensus octamer element and includes OCA-B-DNA contacts (Babb *et al.*, 1997; Chasman *et al.*, 1999). Recent studies have identified a second process-specific coactivator, OCA-S that is involved in the S-phase, cell-cycle dependent stimulation of the histone H2B promoter by Oct-1. Strikingly, this coactivator contains the enzyme GAPDH that may link cellular metabolism to cell growth and division (Zheng *et al.*, 2003). These differential interactions illustrate the structural versatility in Oct-1 protein interactions.

(v) Oct-1 also plays a role in the stimulation of DNA replication as exemplified by the binding of the adenovirus pTP-Pol to the POU-homeobox (Coenjaerts *et al.*, 1994; Phillips and Luisi, 2000). The interaction enhances the association of the pTP-pol with the origin-binding complex. In this case, the interactions are via the DNA binding surface of the

Fig. 8.2. (*continued*) *VP16*: The structure and protein interactions of VP16 are represented. The core structure contains the clustered residues that are critical for the assembly of the IE enhancer complex (HCF-1, Oct-1, DNA) while the transactivation domain (TA, aa 412–490) interacts with a number of basal factors and chromatin modifying components. A schematic representation of the VP16 protein structure is shown (left) indicating the various protein interaction surfaces oriented in recognition of the Oct-1 POU-homeobox/ DNA complex.
HCF-1: The amino-terminal kelch, mid-aminoterminal, proteolytic processing (PPD), autocatalytic (Auto), transactivation (TA), WYF-rich, FN3 repeat, and nuclear localization signal (NLS) regions are represented. The proteins that interact with each region are listed below the appropriate domain. The PPD is represented as a series of consensus (large oval) and divergent (small oval) reiterations of the HCF-1 cleavage sequence shown above. (Bottom left) A stylized representation of the HCF-1 kelch domain is shown illustrating the seven predicted blades (antiparallel sheets, E1 through E4; loops, L1–2 through L4–1). For HCF-1, the predicted ring closure utilizes E4 from the animoterminus and E1-2-3 from the carboxyterminus of the domain (NH2 closure). (Bottom right) The derived molecular model of the HCF-1 kelch domain structure is depicted.
Sp1: The inhibitory domain (INH), transactivation domains (TA-1, TA-2), and DNA binding domains (C2H2, Zn fingers) are represented. Proteins or protein complexes that interact with Sp1 are listed. The structure of the C2H2 Zn finger domain is schematically represented: C, cysteine; H, histadine; F/Y, phenylalanine or tyrosine; y, hydrophobic residue. Light circles represent amino acids that are predicted to make DNA contacts.
GABP: The α subunit contains the ets DNA binding domain recognizing the GA box and the heterodimerization domain (α/β). The β subunit contains ankyrin repeats (α/β heterodimerization region), nuclear localization signals (NLS), transactivation domain (TA), and tetramerization sequences (β−β). The sequence of the transactivation domain is shown and the residues that are critical for both transactivation and interaction with HCF-1 are boxed. (See color plate section.)

POU-homeobox and are distinct from those of the VP16-Oct homeobox complex (de Jong *et al.*, 2002).

Clearly there are a number of distinct mechanisms by which Oct-1 may regulate the basal level expression, induction, or repression of target genes via interactions with specific sites, transcription factors, and coactivators that are mediated through the POU domain. In addition, the protein contains two transactivation domains (Fig. 8.2-Oct-1) that flank the POU domain and function in a promoter context dependent manner, suggesting that additional protein interactions at a given promoter further modulate the regulatory process (Tanaka *et al.*, 1992). The multitude of promoter targets and protein interactions and the diverse regulatory mechanisms involving Oct-1 is reflective of an innate flexibility of the protein conferred by the linker segment separating the POU subdomains (Phillips and Luisi, 2000; Wysocka and Herr, 2003). This flexibility allows for the recognition of sequences and contexts that are divergent from the consensus octamer element. As illustrated by binding to both octamer + and octamer – TAATGARAT elements, the flexibility of the Oct-1 POU domain allows for alternative configurations or positions of the POU-specific box relative to the POU-homeobox (Fig. 8.2-Oct-1). This ability allows for interaction of these domains with proteins in nonconsensus configurations. The configuration is dictated by the sequence of the DNA element and can provide distinct interfaces for various coregulators that are promoter dependent.

The activity of Oct-1 is also regulated by a number of post-transcriptional mechanisms including: (i) enhancement of DNA-binding activity following UV-induced DNA damage (Zhao *et al.*, 2000); (ii) phosphorylation by PKA, PKC, and CKII (Grenfell *et al.*, 1996); (iii) interaction with HMG2 which may function to order the DNA-binding domain for high affinity recognition (Zwilling *et al.*, 1995) and (iv) enhanced phosphorylation of Oct-1 via interaction with MAT1 cyclin dependent kinase activating kinase (Inamoto *et al.*, 1997). Similarly additional levels of regulation exist which impact the function of Oct-1 as illustrated by the modulation of the levels of the OCA-B coactivator by the ring finger protein Siah-1 (Tiedt *et al.*, 2001). The various types of interactions and levels of regulation that impinge on the function of Oct-1 will clearly have implications for the function of the protein in the stimulation of the HSV IE genes in different cellular contexts.

VP16

Vp16 (ICP25, VMW65, αTIF) is the HSV-encoded component of the enhancer core complex that determines the specificity for the HSV elements. Approximately 900 molecules of the protein are packaged within the tegument structure of a virion and are released into the cytoplasm of the cell upon infection (Roizman, 1996). The protein is transported to the nucleus and assembled into the stable enhancer core complex. VP16 is a critical component of the complex as it determines the specificity of the HSV IE core complex by direct recognition of the TAATGARAT DNA element and by selective recognition of the DNA bound Oct-1 (Figs. 8.1 and 8.2) (Kristie and Sharp, 1990; Lai *et al.*, 1992; Pomerantz *et al.*, 1992).

The 490 amino acid protein consists of a conserved structural core (aa 49–403) that contains the specificity surfaces for interaction with Oct-1, DNA, and HCF-1 (Lai and Herr, 1997; Simmen *et al.*, 1997; Wysocka and Herr, 2003) as well as a highly characterized transcription activation domain (aa 412–490) that interacts with both basal transcription factors and chromatin remodeling/nucleosome modification factors (Fig. 8.2-VP16). Surprisingly, the protein lacks a nuclear localization signal and nuclear transport is provided by protein interaction with HCF-1 (La Boissiere *et al.*, 1999). As HCF-1 is a key cell-cycle component, this interaction may play a significant role in the initial "sensing" of the cell state for viral replication.

Advances in the determination of the structure of VP16 have provided an understanding of the protein–protein and protein–DNA interactions that are critical for the protein's selective induction of the HSV IE genes. The highly structured core domain consists of internal symmetry that is dominated by two-stranded antiparallel coiled coils. The resulting structure resembles a "seat" complete with bottom, back, and headrest regions (Fig. 8.2-VP16). Interestingly, the region representing the interaction domain for Oct-1, HCF-1, and DNA (aa 350–394) is disordered in the crystal, suggesting that this domain adopts a structure upon binding these components (Babb *et al.*, 2001; Liu *et al.*, 1999). Correlation of the structure with numerous mutagenesis studies clearly indicates that there are distinct surfaces for interaction with components involved in virion assembly (right surface) vs. transcription (left surface) while the DNA recognition surface is formed by a cleft in the seat structure (Fig. 8.2-VP16, bottom right) (Babb *et al.*, 2001; Liu *et al.*, 1999). As different VP16 orthologs such as those encoded by VZV and BHV recognize different TAATGARAT elements dependent upon the 3′ sequences, the VP16 structure presents a model by which the unstructured region may also contribute to DNA binding by adopting a structure to specifically recognize the GARAT sequences while the "seat" recognizes the 3′ portion of the element. Furthermore, the DNA binding model predicts that the Oct-1 POU-specific box may lie to either side of the TAAT in the enhancer complex

(refer to Fig. 8.2 -VP16) and may modulate the VP16-DNA interaction.

The specific residues that are important for the interactions of VP16 in the IE enhancer core complex have been defined by numerous mutagenesis and peptide inhibition studies (Vogel and Kristie, 2001; Wysocka and Herr, 2003). This work elucidated a clustered core of residues for interaction with HCF-1 (E361, H362, Y364) that is now recognized as the HCF-1 interaction (D/E HXY) motif in a number of cellular proteins that interact with this coactivator protein (Freiman and Herr, 1997; Lu *et al.*, 1998; Wysocka and Herr, 2003). Mutations in residues (Y373, G374, S375) affect the VP16- Oct1 interaction while mutations in residues (R360, R366, R368, K370) affect DNA binding (Lai and Herr, 1997). Interestingly, S375 is a target for CKII-dependent phosphorylation. This residue is normally modified in cell extracts and is required for efficient complex formation, suggesting post-transcriptional modulation of the VP16 interactions required for the IE complex assembly (O'Reilly *et al.*, 1997).

The second unstructured region in the crystal determination is the transcription activation domain (TA, aa 412–490) that is located at the "bottom" of the determined structure (Liu *et al.*, 1999). The VP16 TA domain has been perhaps the most utilized tool in studies of the mechanisms involved in transcription activation. Initially, mutagenesis studies defined the TA domain and determined that this region consisted of two distinct subdomains containing important aromatic and hydrophobic residues (Regier *et al.*, 1993). The effects of substitutions at these positions also suggested that the structure of these subdomains were distinct and were likely to be involved in different stages of transcription stimulation. The unstructured nature of the domain both in solution and in the crystal determination suggests that this region also becomes structurally constrained upon protein-protein interactions (Shen *et al.*, 1996).

Many studies on the mechanisms of transcription activation have utilized the VP16 TA domain, generally in the context of a DNA binding domain fusion protein. These studies have elucidated protein interactions and general principles or mechanisms in the regulation of transcription activation. While early studies demonstrated that activators promote "open complex" formation by enhancing the formation of the RNAPII preinitiation complex and promoter assemblies; later analyses addressed the rate limiting stages, protein interactions, and stepwise activation stages that were affected by the TA domain in given promoter contexts. The data from many such studies have resulted in general models for promoter activation via multiple steps which are not necessarily strictly ordered but can be affected by activators including: activator access and binding, histone modification and chromatin remodeling, binding and assembly of the basal factor-RNAPII complexes, open complex formation, RNAPII promoter escape, RNAPII pausing, transcription elongation, coupled transcription-mRNA splicing, and transcription reinitiation (Cosma, 2002).

Studies that focus upon protein interactions of the VP16 TA domain have suggested that the unstructured domain adopts conformations upon binding specific targets. This flexibility allows for numerous conformations and sequential interactions with factors involved in different stages of promoter activation (basal factors TFIIB, TFIIH, TBP; histone modification SAGA; and chromatin remodeling SWI2/SNF2) (Gold *et al.*, 1996; Hall and Struhl, 2002; Herrmann *et al.*, 1996; Herrera and Triezenberg, 2004; Krumm *et al.*, 1995; Memedula and Belmont, 2003; Nishikawa *et al.*, 1997; Vignali *et al.*, 2000; Walker *et al.*, 1993; Xiao *et al.*, 1994; Yudkovsky *et al.*, 2000). Consistent with early studies, the two subregions of the TA domain interact with distinct factors and function in multiple steps or activation stages including: (i) the ATP-dependent chromatin remodeling and histone modifications that allow factor access and promoter targeting (SWI, SAGA, p300) as demonstrated by the ability of the TA domain to mediate large scale chromatin remodeling; (ii) the assembly of the RNAPII preinitiation complex by alleviating TBP autorepression or by direct recruitment of the protein followed by competition with TBP-basal TAFs interactions to promote enhanced activation; (iii) recruitment of TFIIB leading to RNAPII positioning and open complex formation; (iv) recruitment of TFIIH and CTD kinases that mediate RNAPII promoter escape; (v) regulation of the efficiency of the initiation complex formation that functions to increase the elongation competency of the complexes, thereby affecting the coupled mRNA splicing efficiency; and (vi) stabilization of the reinitiation scaffold (TFIID, TFIIA, TFIIE, mediator and TFIIH) via interactions with TFIIH, leading to efficient reassembly and reinitiation.

These studies have resulted in significant advances in the understanding of general transcription initiation and promoter activation and have suggested mechanisms by which the VP16 TA domain may participate in the activation of the IE gene transcription. It remains, however, to be determined exactly what the inherent rate-limiting steps are for the activation of the IE genes and what the contribution(s) of the VP16 TA domain are within this context. In addition, clearly the contributions of all of the core and ancillary factors will be affected by the interplay of the regulatory proteins and signals within a given cellular milieu.

HCF-1

The last required component for the stable assembly of the core enhancer protein complex is the cellular coactivator HCF-1. Originally identified as a required component derived from extracts of insect or mammalian cells for the formation of a stable core complex in vitro, HCF-1 was subsequently biochemically purified and the gene encoding it was cloned (Kristie *et al.*, 1989, 1995; Kristie and Sharp, 1993; Vogel *et al.*, 2001; Wilson *et al.*, 1993; Wysocka *et al.*, 2003). Interestingly, rather than a single polypeptide, the protein is actually a family of polypeptides ranging from 68–230 kD that are derived from a common precursor via site-specific proteolysis (Kristie *et al.*, 1995; Vogel and Kristie, 2000a; Wilson *et al.*, 1993). The protein is ubiquitously expressed and localized in the nucleus of all cell types with a notable exception (discussed below). Numerous studies in recent years have illuminated both the functions of the protein in HSV IE gene expression as well as its functions in basic cellular processes. The importance of both lies in the strict requirement for HCF-1 in the initiation of HSV lytic cycle as well as the importance of the protein in several basic cellular processes that may impact the viral cycle.

As shown in Fig. 8.2, multiple functional domains and protein interactions have been defined which have identified HCF-1 as a critical component of processes such as cell-cycle control, positive and negative transcription regulation, chromatin modulation, DNA replication, and mRNA splicing. Many studies have focused upon the amino terminal domain of the protein as this region is required for the formation of the HSV IE enhancer core complex as well as for cell cycle progression (Goto *et al.*, 1997; Hughes *et al.*, 1999; LaBoissiere *et al.*, 1997; Wilson *et al.*, 1997). The predicted structure of the amino-terminus of HCF-1 is based upon structural alignments to related "kelch" domain proteins (Adams *et al.*, 2000; Wilson *et al.*, 1997) and a molecular model has been derived based upon the crystal structure of galactose oxidase "kelch" domain (Fig. 8.2 -HCF-1, bottom right) (J.L. Vogel and T.M. Kristie, unpublished data). The domain model consists of seven reiterations of four antiparallel β sheets that form the blades of a propeller-type structure. The ring is closed via the E4 sheet of the aminoterminus with the E1-E2-E3 sheets at the carboxyterminus of the domain to form the 7th blade (Fig. 8.2-HCF-1, bottom left). Kelch domain proteins are involved in a broad range of functions from structural assemblies to signal transduction and the domain presents several distinct protein interaction surfaces formed by the seven L2-3 loops, the L4-1 loops, and the E4 sheets (Adams *et al.*, 2000).

As defined in numerous studies, the kelch domain mediates the high affinity interaction of HCF-1 with VP16 in the assembly of the HSV IE enhancer complex. The analysis of this interaction led to the elucidation of the HCF-1 interaction motif (D/E HXY) recognized in VP16 and subsequently determined to be a common motif found in cellular proteins that interact with the HCF-1 kelch domain (Freiman and Herr, 1997; Lu *et al.*, 1998; Simmen *et al.*, 1997). However, selective mutagenesis has suggested that distinct surfaces within the reiterated kelch structure are involved in the interactions that are mediated by this short common motif (Mahajan and Wilson, 2000). Differences between HCF-1 and the highly related HCF-2 in blades 5 and 6 encode part of the specificity for the preferential binding of VP16 to HCF-1 in a manner analogous to the discrimination between Oct-1 and Oct-2 (Johnson *et al.*, 1999). While the kelch domain is the minimal domain required for the assembly of the VP16 enhancer core complex, it is unlikely to represent the only domain involved in this assembly. Some studies have suggested that additional domains are required, perhaps to constrain or alter the positioning of the VP16 activation domain for stable assembly into the complex (La Boissiere *et al.*, 1997). Notably, multiple other cellular proteins that interact with the kelch domain have also been isolated including transcription factors (LZIP, Zhangfei) E2F1, E2F4, Krox 20 (Freiman and Herr, 1997; Knez *et al.*, 2006; Lu and Misra, 2000b; Lu *et al.*, 1997; Luciano and Wilson, 2002, 2003), transcription coactivators (PGC, PRC) (Lin *et al.*, 2002), a nuclear export protein (HPIP) that may control the nucleo-cytoplasmic pool of HCF-1 (Mahajan *et al.*, 2002), and chromatin modification components (set1/Ash HMT) (Wysocka *et al.*, 2003); suggesting that the protein is involved in numerous or global cellular transcription functions.

In its role during the initial stages of the HSV lytic cycle, the protein is described as the coordinator of the HSV IE enhancer complex assembly as it has interactions with each of the enhancer components (VP16, Oct-1, Sp1, GABP) and may orient the assembled factors for effective activation of the IE gene transcription (Vogel and Kristie, 2000b; Vogel *et al.*, 2001). The interactions may also reflect a central role in the activation of the IE transcription in response to multiple distinct regulatory signals that are mediated by the various factors involved (see below). An additional function of the protein in the regulation of the IE genes is evidenced by its activity as a mediator or coactivator of transcription via interaction with various transcription factors, chromatin modification components and other coactivators (Fig. 8.2 -HCF-1). Several lines of evidence support the proposal that HCF-1 can function as

a general coactivator and mediates transcription activation of the assembled IE complex including: (i) an activation domain in the carboxyterminus of HCF-1 functions synergistically with the VP16 TA domain and may affect a distinct rate-limiting stage (Luciano and Wilson, 2002); (ii) HCF-1 is required to mediate the transcriptional activation potential of LZIP at CRE sites (Lu *et al.*, 1997); (iii) HCF-1 interacts directly with the TA domain of GABP where mutations which affect the transactivation potential correlate directly with affects upon the GABP-HCF-1 interaction (Vogel and Kristie, 2000b); (iv) HCF-1 interacts with chromatin modifying proteins presumably to recruit these enzymes in early stages of transcription activation (Wysocka *et al.*, 2003); and (v) depletion of HCF-1 results in ablation of HSV 1E expression (Narayanan *et al.*, 2005). Significantly, HCF-1 dependent transcription events such as defined for GABP can provide a VP16-independent alternative mechanism for the induction of the HSV IE gene expression outside of the TAATGARAT element core.

In addition to its role in the direct assembly of the IE enhancer core complex, studies delineating the HCF-1 NLS (aa 2015–2035) have demonstrated that HCF-1 is required for the nuclear transport of VP16 during productive infection (La Boissiere *et al.*, 1999). These results suggest that the pool of HCF-1 that is utilized by HSV is likely to be free cytoplasmic protein and that preassociation with VP16 is a critical stage in the enhancer complex assembly. Proteins such as the nuclear export factor HPIP, which interacts with the kelch domain of HCF-1, may play a role in regulating the nucleo-cytoplasmic shuttling of the protein, can therefore have a significant regulatory impact on the availability of cytoplasmic HCF-1 for the HCF-1/VP16 interaction and transport.

HCF-1 is also a critical control component of the cell-cycle as initially demonstrated by the isolation of a ts mutant (P134S) that resulted in G0-G1 cell cycle arrest (Goto *et al.*, 1997). While the exact mechanism(s) of HCF-1 dependent cell-cycle progression remain unclear, studies indicate that the protein has multiple roles in promoting several cell-cycle stages: (i) the dissociation of ts-HCF-1 from cell chromatin at the non-permissive temperature suggests a global effect on cellular transcription (Wysocka *et al.*, 2001); (ii) the interaction of HCF-1 with the TA domain of Miz-1 results in repression of Miz-1 activation of cdk $p15^{INK4b}$ expression, potentiating cell cycle progression (Piluso *et al.*, 2002); (iii) the WYF domain (aa 1793–2005) in the carboxyterminus of HCF-1 interacts with PDCD2 which can suppress complementation of growth arrested ts-HCF-1 cells suggesting that PDCD2 may negatively regulate HCF-1 functions possibly through the association with additional transcription repression components such as NcoR (Scarr and Sharp, 2002); (iv) RNAi-mediated depletion of HCF-1 results in defects in both G_0–G_1 progression and cytokinesis/exit from mitosis which can be rescued by expression of the HCF-1 aminoterminus or carboxyterminus, respectively (Julien and Herr, 2003; and (iv) array studies have implicated HCF-1 in the expression of critical cellular proteins involved in general transcription, cell cycle progression, DNA replication-repair, and signal transduction (Khurana and Kristie, 2004). These studies collectively suggest that HCF-1 regulates cell cycle progression through its multiple roles in the regulation of gene expression. Interestingly, observations that novel HCF-1 aminoterminal polypeptides accumulate in the cytoplasm of cells arrested in G_0 may indicate that specific subdomains of the protein are localized in a regulated manner to control cell-cycle progression (Scarr *et al.*, 2000).

Located in the central region of the 230 kD HCF-1 precursor is one of the more unusual domains consisting of a series of 20 amino acid reiterations that are sites of the specific proteolytic cleavages that results in the family of HCF-1 amino and carboxyterminal polypeptides (PPD domain, Fig. 8.2 -HCF-1). However, the amino and carboxyterminal proteins do not segregate but remain tightly associated via interactions between fibronectin type II repeats in the carboxyterminus (aa 1800–2000) and the 7^{th} blade of the kelch domain (Kristie *et al.*, 1995; Kristie and Sharp, 1993; Wilson *et al.*, 2000). The processing of the protein has been determined to be autocatalytic and requires the PPD and residues carboxyterminal to this domain for efficient processing in vitro (Vogel and Kristie, 2000a). The functional role of the processing remains elusive but may represent a regulatory mechanism for controlling HCF-1-protein interactions and amino-carboxyterminal cooperativity. A novel regulatory function is proposed by the identification of a series of protein-protein interactions within the PPD in which specific reiterations encode an inherent specificity for particular protein binding partners. This model predicts that processing of HCF-1 would regulate the ability of the resulting cleavage products to interact with a specific subset of cofactors, thereby determining the activity of the particular HCF-1 cleavage product (Vogel and Kristie, 2006). Ultimately, the processing may control the protein's nuclear transport, cell-cycle functions, and/or determine its transcription activation or repression potential.

While the emphases of HCF-1 studies have been the roles of the protein in transcriptional regulation and cell cycle progression, the protein has also been implicated in mRNA splicing where it may be a general cofactor (Ajuh

et al., 2002) and in protein modification complexes (HCF-1-Protein Phosphatase 1) (Ajuh *et al.*, 2000) where it may regulate the activity of the phosphatase or determine it's targets. In addition, HCF-1 may play roles in the later stages of HSV lytic replication as suggested by the interaction of the kelch domain with HSV DNA replication proteins (Peng M.L., Nogu eira, and T.M. Kristie 2006, unpublished data).

Clearly HCF represents one of the more complex components of the HSV IE regulatory assembly and has diverse and essential cellular functions. This protein may also be the component that is most critical for viral IE expression suggesting that the evolution of the virus to usurp the functions of this factor has more implications for the regulation of the HSV lytic cycle than is initially readily apparent.

Ancillary factors: Sp1 and GABP

In addition to the enhancer core complex, each IE regulatory domain contains a number of binding sites for cellular transcription factors such as Sp1 and GABP. The expression of the IE genes, even in the absence of VP16, attests to the significance of these components. However, the potential of these elements are dependent upon and enhanced by the presence of the assembled IE enhancer core complex, reflecting an interdependence of the elements.

Sp1

Sp1 is a member of the Zn finger/krupple family and was the first transcription factor to be purified and cloned (Black *et al.*, 2001; Kaczynski *et al.*, 2003; Suske, 1999). The protein recognizes the element GGGCGG (GC box) that is present in multiple copies in the HSV IE regulatory domains (Fig. 8.1) and has been determined to contribute to the basal expression level (Jones and Tjian, 1985). Originally considered to be a ubiquitously expressed, housekeeping factor, the protein is now known to be a member of a large family of related proteins that are subject to and involved in distinct regulatory pathways. Sp1 through Sp6 are highly related proteins which all interact with the GC element although there is some variability in specific binding affinity.

Sp1 is a 778 amino acid protein consisting of a three C2H2 Zn-finger DNA binding domain (C-X_{2-5} C-X_3-(F/Y)-X_5-ψ-X_2-H-X_{3-5}-H) located in the carboxyterminus with an embedded nuclear localization signal (NLS, Fig. 8.2-Sp1). Models that predict the DNA binding contacts are based upon similar structures and suggest that key residues in the various Sp family members determine the specificity for the GC box (Kaczynski *et al.*, 2003). The protein also contains two TA domains [(S/T) and Q-rich] that function in a promoter-dependent context, conceptually similar to those in Oct-1 (Black *et al.*, 2001; Suske, 1999).

Sp1 interacts with a number of known transcription activators (e.g., Oct-1), transcription coactivators (e.g., HCF-1, p300/CBP), and basal transcription factors (e.g., TBP)(Gunther *et al.*, 2000; Kaczynski *et al.*, 2003; Suske, 1999). In a manner analogous to Oct-1, the protein interactions and functional significance can largely be determined by the promoter context. Most significantly, the accumulating data illustrates that this family of proteins is subject to several regulatory signals and pathways in which specific GC boxes mediate responses to particular stimuli via binding proteins involved in those pathways. In addition, the particular regulatory response of a given GC box can also vary depending upon the particular cell context. These proteins interact with factors such as Rb-p107, p53, E2F, and Oct-1 and respond to signals as diverse as growth stimuli, NGF, TGFb, hormones, DNA damage, and apoptosis (Black *et al.*, 2001; Gunther *et al.*, 2000; Kaczynski *et al.*, 2003; Ryu *et al.*, 2003; Suske, 1999; Yan and Ziff, 1997). An additional level of regulation is provided by varying levels of the Sp family within a given cell type and under specific response conditions. As Sp1 and Sp3 are expressed in the same cell types, bind with equal affinity to the GC box, and have distinct functions; the ratio of the proteins can determine the activation vs. repression of given target genes. This regulatory mechanism is exemplified by alterations in the ratios of these proteins during cell differentiation or specific signaling pathways (Black *et al.*, 2001; Gunther *et al.*, 2000; Kaczynski *et al.*, 2003; Suske, 1999). The activation and repression functions of the Sp family are hypothesized to be mediated via interactions with HAT or HDAC complexes, respectively.

Finally, as expected by the involvement of these proteins in response to environmental signals, modifications of the factors also modulate that activity of the family. For example, acetylation of Sp1 in response to neuronal oxidative stress plays a role in neuronal survival pathways (Ryu *et al.*, 2003). In an HSV infection, phosphorylation of Sp1 later in infection decreases the protein's TA potential and may contribute to the down regulation of the IE gene expression (Kim and DeLuca, 2002).

GABP

GA rich sequences adjacent to at least one TAATGARAT element in each of the HSV IE regulatory domains were originally identified in mutagenesis studies and reporter assays where these elements contributed to the VP16-dependent induction of IE expression (Triezenberg *et al.*, 1988). In vitro transcription assays further demonstrated the

significance of the elements for VP16-mediated transcription and suggested that the sites functioned synergistically with the enhancer core (Wu *et al.*, 1994).

The factors binding to these elements (GABP) are related to the ets and notch protein families and consist of an α-subunit containing a carboxyterminal ets DNA binding domain and a β subunit containing the TA domain, NLS, and series of ankyrin repeats that mediate dimerization with the α subunit as well as contributes to DNA binding (Fig. 8.2-GABP) (LaMarco *et al.*, 1991; Thompson *et al.*, 1991). The proteins also form tetrameric structures via carboxyterminal sequences in the β subunits forming helical intertwined coiled coils (de la Brousse *et al.*, 1994). In the β-subunit, the TA domain consists of a series of hydrophobic clusters which are critical for the TA potential (Gugneja *et al.*, 1996; Gugneja *et al.*, 1995). These regions correlate with the ability of the domain to interact with the enhancer core coactivator HCF-1, indicating that HCF-1 mediates the activation potential of the factor (Vogel and Kristie, 2000b).

The focus of many GABP studies has been in elucidating the role of the protein in the activation of nuclear encoded mitochondrial respiration component and assembly genes (Puigserver and Spiegelman, 2003; Scarpulla, 2002). These studies have delineated multiple levels of regulation and have indicated that an important mechanism for regulation of these factors is via regulation of the coactivators which respond to a variety of stimuli resulting in phosphorylation, induction, activation, and stabilization of these coactivators. The coactivators subsequently recruit additional cofactors, coactivators, and histone modification complexes. Two coactivators have been intensely studied in this context: PRC that mediates activation in proliferative responses and PGC that mediates thermogenic effector responses (Scarpulla, 2002). Interestingly, PGC has not been shown to directly interact and modulate GABP activity but may, in fact, do so via its interaction with the kelch domain of HCF-1 (Fig. 8.2-HCF-1). The activity of GABP and its cofactors are modulated by several signaling pathways such as p38 MAPK. For PGC, p38 mediated phosphorylation results in stabilization of the cofactor (Puigserver and Spiegelman, 2003). The phosphorylated PGC is also involved in direct induction of the expression of GABP.

Numerous regulatory response pathways are mediated by GABP including (i) induction of neuregulin expression (Fromm and Burden, 1998); (ii) insulin dependent prolactin gene expression via MAP pathway phosphorylation of GABP (Ouyang *et al.*, 1996); (iii) TPA stimulated IL2 induction which is mediated via JNK activation of GABP (Hoffmeyer *et al.*, 1998); (iv) the HIV LTR induction mediated via Raf-1 kinase activation of GABP (Flory *et al.*, 1996) and (v) the MMTV LTR which is synergistically activated by GABP in the presence of glucocorticoids (Aurrekoetxea-Hernandez and Buetti, 2000). The protein is also involved in numerous regulatory events by synergistic interactions with other transcription factors such as Sp1 and the cAMP responsive proteins CREB and ATF (Bannert *et al.*, 1999; Sawada *et al.*, 1999).

VZV IE gene expression: parallels and divergence

This review has focused on the regulation of HSV IE genes as a representative model of the mechanisms involved in the IE expression of an alpha herpesvirus due to the focus of studies in this area. In contrast, little has been elucidated concerning the mechanisms involved in the regulation of VZV IE gene expression. What has been determined, however, shows striking parallels to the HSV model. In VZV, the expression of the IE gene (IE62) is controlled by an enhancer domain (Fig. 8.3) consisting of multiple defined elements (Bannert *et al.*, 1999; McKee and Preston, 1991; Moriuchi *et al.*, 1995; Sawada *et al.*, 1999).

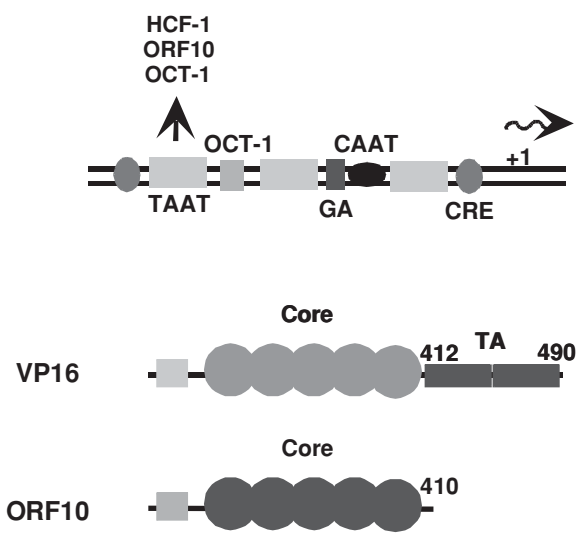

Fig. 8.3. The VZV IE62 regulatory domain. (Top) The arrangement of elements that have been delineated in the VZV IE62 regulatory region are indicated (TAAT, enhancer core element TAATGARAT; Oct, Octamer element; GA, GABP binding site; CAAT, CAT box; CRE, c-AMP responsive element). The assembly of the components HCF-1, Oct-1, and the VZV VP16 ortholog ORF-10 on the TAAT element is indicated. (Bottom) Comparison of the domain structure of HSV VP16 and VZV ORF-10 illustrates conservation of the aminoterminus and core domain and the absence of an ORF-10 transactivation domain.

Similar to HSV, the domain contains reiterations of a TAATGARAT element that is important for induction via the VZV virion component ORF10, the ortholog of the HSV VP16 (Moriuchi *et al.*, 1995). Additional elements include a GABP binding site and c-AMP responsive sites (CRE) that also contribute to the ORF10-mediated stimulation of the IE62 gene expression. As has been demonstrated for HSV, the VZV IE62 TAATGARAT elements can nucleate the assembly of enhancer core complexes that contain Oct-1 and HCF-1 in concert with the ORF10 protein (Moriuchi *et al.*, 1995).

The VZV ORF10 transactivator, while sharing significant homology to VP16, exhibits a striking difference that impacts the regulatory process. In contrast to VP16, ORF10 does not contain a transactivation domain, indicating that the interaction of the protein with other factors and coactivator components (e.g., Oct-1, HCF-1) must provide this function. A second significant difference between the regulatory mechanisms involved in the induction of HSV IE and the VZV IE genes is the autoregulatory response mediated by the major IE gene products (Perera *et al.*, 1992). In HSV, α4/ICP4 functions to down regulate the IE genes in the transition from IE to E gene expression (Roizman, 1996). In VZV, IE62 functions to induce or enhance its own transcription. Interestingly, IE62 is also a component of the virion tegument structure (Kinchington *et al.*, 1992) and may be a significant component of the IE induction response, thus compensating for the lack of the transactivation potential of the ORF10 activator. However, despite minor variations, data on the regulation of the VZV IE62 gene closely parallels the components and mechanisms defined in the regulation of HSV IE genes.

Regulation of the IE genes: multiple levels and response potentials

Studies on the regulation of the IE genes and the various components involved have led to advances in the understanding of enhancer complexes, ordered assembly processes, protein surfaces and interactions, mechanisms of transcriptional activation, and orders of interplay regulation. In addition to the modulation of DNA site recognition, the various transcription factors themselves are subject to modifications, alterations in turnover rates, subcellular localization, and signaling response pathways. Transcription factor synergy (activation or repression) is also dependent upon the cellular milieu and the balance of factors including the availability, competition, activator/repressor ratios, and cell-specific cofactors and coactivators. Additional higher orders of regulation are dependent upon the regulation of the coactivator levels, interactions, and functions. The ability and efficiency of the various assembled complexes to circumvent or alter rate limiting stages in the transcriptional assembly, initiation, elongation, and reinitiation is also likely to depend upon the cell type and state as the consequences of chromatin/nucleosomal structure, available factors, and signal environment will impinge upon a given rate limiting stage and determine the requirements for efficient transcription.

Most strikingly, the complex interactions of the components involved in the regulation of the IE enhancer complexes (e.g., Oct-1, Sp1, GABP, HCF-1) and the ability of these components to respond differentially to multiple environmental signals point to the evolution of the IE regulatory domains to respond to diverse signals.

The regulation of the IE genes: reactivation of HSV from the latent state

The components expressed in specific cell types may impact the normal lytic cycle regulation as suggested by the low levels of Oct-1 expression in sensory neurons. In this situation, other POU proteins such as Oct-2 or members of the Brn family may play a role in suppression or inefficient activation of the IE gene (Brownlees *et al.*, 1999; Latchman, 1996; Latchman, 1999; Lillycrop *et al.*, 1994). For the Brn family, Brn3a functions as an activator while Brn3b functions as a transcriptional repressor and the relative levels of these proteins change upon differentiation or neuronal stimulation (Latchman, 1999). Similarly, the levels of Sp1/Sp3 provide potential for repressor assemblies that may play a role in modulating IE expression in neuronal cells.

The potential of the IE gene domains to respond to diverse environmental stimuli suggests that these domains may respond to latency-reactivation stimuli by targeting distinct elements or components. Significantly, each of the identified IE regulatory factors is commonly linked by its interaction with the HCF-1 coactivator. Therefore, while distinct signals and factors may respond to distinct stimuli, the critical response component may be represented by the common coactivator. Interestingly, HCF-1 itself is uniquely sequestered in the cytoplasm of sensory neurons and is rapidly transported to the nucleus in response to reactivation signals (Fig. 8.4, Top) (Kristie *et al.*, 1999). This regulated transport may well reflect an important element of the activation of the IE genes during the reactivation process. Furthermore, this provides a mechanism for the induction of the IE gene expression in response to signaling events in a VP16-independent manner, utilizing com-

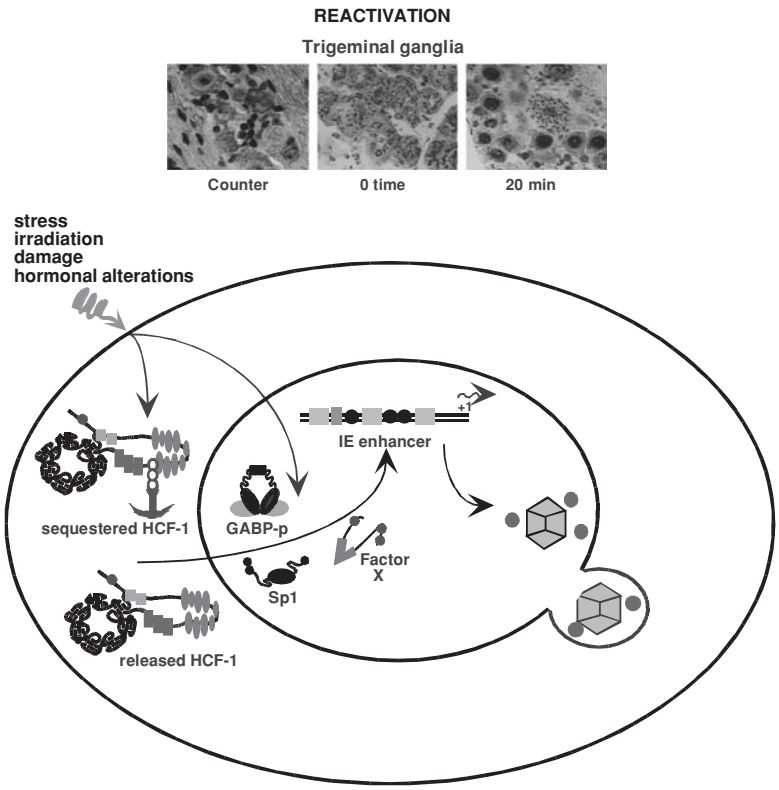

Fig. 8.4. Model of the induction of the IE genes during HSV reactivation from latency. (Top) Immunohistochemistry studies of HCF-1 demonstrate that the protein is specifically sequestered in the cytoplasm of sensory neurons (0 time, middle panel) and rapidly transported to the nucleus under experimental conditions that reactivate HSV from latency (explant reactivation stimuli, right panel). (Bottom) Schematic depiction of the activation of IE enhancer components during the initiation of reactivation. Environmental signal(s) result in the release of cytoplasmically sequestered HCF-1 and the activation of DNA binding factors such as GABP, Sp1, or other factors which function in concert with HCF-1 to activate the IE genes and initiate the viral lytic cycle. (See color plate section.)

plexes such as GABP-HCF-1 and Sp1-HCF-1. In support of these potential mechanisms, studies in Oct-1 knockout cells have demonstrated that the protein is important but not essential for IE expression, thereby suggesting that alternative complexes and factors can initiate the cycle (Nogueira *et al.*, 2004). In contrast, IE expression is not detected in cells depleted of HCF-1 (Narayanan *et al.*, 2005). As proposed in Fig. 8.4, Sp1 and or GABP may be involved in the activation of the IE genes during the reactivation process. However, other factors may be involved as suggested by the activation of ICP0 transcription via L-ZIP. L-ZIP, like HCF-1 exhibits a cytoplasmic sequestering and may also respond to signals that result in reactivation (Lu and Misra, 2000a). It is interesting to speculate that multiple reactivation pathways are possible utilizing distinct subsets of factors that respond to distinct signals.

Questions and future directions

Much has been learned in recent years and as is often the case, new information leads to many new questions and directions. Future studies certainly will extend the work established by the identification of the critical components of the HSV IE transcription complexes. Continuing assessment of the functions of these components in chromatin modulation, alteration of rate limiting stages and transcriptional mechanisms will provide additional basic process information. Determination of the regulation of each component within varying cellular contexts as well as the development of genetic animal models can delineate the response and impact of the factors subject to various signal pathways and stimuli. Protein modifications, localization dynamics, regulation of turnover, and the relationship of the components within a given context are key

areas for the determination of the components which may play central roles in the HSV lytic, latent, and reactivation processes.

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9

Initiation of transcription and RNA synthesis, processing and transport in HSV and VZV infected cells

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Initiation of transcription and RNA synthesis

The alphaherpesviruses, HSV-1 and VZV encode TATA-box containing promoters that are transcribed by the cellular RNA polymerase II

During productive infection by herpes simplex virus type 1 (HSV-1), approximately 80 genes encoded within the linear 152-kbp viral genome are expressed in three sequential phases that are termed immediate early (IE; α), early (E; β) and late (L; γ) (Honess and Roizman, 1974; McGeoch, 1991). The smaller, 125-kbp varicella zoster virus (VZV) genome encodes around 70 genes, which are also expressed as IE, E and L products (Davison and Scott, 1986). HSV-1 and VZV genes are transcribed by the cellular RNA Polymerase II and each viral promoter has a TATA box homology about 25 nucleotides upstream of the start site of transcription (for review, see Wagner *et al.*, 1995). In HSV-1infections, the first genes to be transcribed are the five IE genes, which are distinguished from E and L genes by specific sequence elements termed TAATGARAT sequences in the upstream regions of IE promoters. These elements are recognized by a virion tegument protein, VP16, which binds as part of a protein complex that contains two cellular factors, Oct-1 and HCF, to transcriptionally activate expression of IE genes (Wysocka and Herr, 2003). VZV IE genes do not appear to encode upstream promoter elements similar to the TAATGARAT sequence, however VZV does encode a protein, ORF10 that exhibits similarities with VP16, although its activity has been much less well characterized than that of VP16 (Piette *et al.*, 1995).

HSV-1 E promoters, unlike the IE promoters, do not contain VP16-responsive sequence elements, however they do contain a number of *cis*-acting elements upstream of the TATA box that have been shown to bind cellular transcriptional activators, including Sp1 (Kim and DeLuca, 2002; Wagner *et al.*, 1995). In contrast, HSV-1 late promoters lack upstream *cis*-acting sequence elements, and instead the region downstream of the TATA box has been shown to play an important role in regulating the expression of several late genes (Guzowski and Wagner, 1993; Kim *et al.*, 2002; Pederson *et al.*, 1992). The architecture of VZV promoters appears similar to that of HSV-1 promoters, however, binding sites for the cellular transcription factors Sp1 and USF have been found in a large number of potential promoters for VZV genes from all three kinetic classes (Ruyechan *et al.*, 2003).

Viral factors required for the initiation of transcription on HSV-1 and VZV promoters

Like HSV-1 IE genes, which are activated by VP16, the E and L genes also require a virally-encoded transcription factor to induce expression. The factor that is required is one of the IE proteins, termed infected cell polypeptide 4 (ICP4). ICP4 is a nuclear phosphoprotein that acts as a homodimer to activate or repress transcription depending upon the promoter (Shepard *et al.*, 1990). ICP4 is absolutely required for abundant expression of all E and L gene products, and thus it is essential for productive infection (DeLuca *et al.*, 1985). In the absence of functional ICP4, not only are early genes poorly expressed, but some IE genes are overexpressed. The overexpression of IE genes in the absence of functional ICP4 reflects the ability of ICP4 to repress transcription from its own promoter in an autoregulatory fashion, as well as from other IE promoters (Gu *et al.*, 1995; Leopardi *et al.*, 1995). Genetic and biochemical studies have shown that ICP4 binds to DNA. ICP4 represses transcription from specific viral promoters that have a high-affinity ICP4 binding site spanning the transcription initiation site, and it does so by interacting with the basal transcription

factors TATA-binding protein (TBP) and TFIIB (Faber and Wilcox, 1988; Gu *et al.*, 1995; Leopardi *et al.*, 1995). ICP4 also stimulates transcription from early and late viral promoters through interactions with viral DNA and cellular basal transcription factors. However, despite extensive analyses, specific sequences that bind ICP4 with high affinity that are common to all promoters activated by ICP4 have not been uncovered, although the ICP4 DNA binding domain is essential for activation of transcription (Shepard *et al.*, 1989; Smiley *et al.*, 1992). The minimal *cis*-acting elements required for stimulation of transcription by ICP4 are a TATA box and in some late promoters, an initiator element (Cook *et al.*, 1995; Kim *et al.*, 2002). ICP4 facilitates formation of the preinitiation complex through interactions with one or more components of the cellular transcription machinery. ICP4 forms a tripartite complex with TFIIB and TBP on DNA, enhancing the binding of TFIID to DNA (Grondin and DeLuca, 2000). ICP4 also interacts with TBP-associated factor 250 (TAF250), and promotes the formation of transcription preinitiation complexes on promoters (Carrozza and DeLuca, 1996).

A number of genetic and biochemical analyses have been conducted to determine which of the observed interactions between ICP4, viral DNA, and cellular proteins are relevant to the function of ICP4 as an activator. ICP4 is comprised of 1,298 amino acids. It has been found that ICP4 residues 1 to 315 function as a transactivation domain (Xiao *et al.*, 1997); further, this region binds to the cellular EAP protein (EBV EBER-associated protein) (Leopardi *et al.*, 1997), and is required for formation of a complex with DNA that contains TBP and TFIIB (Smith *et al.*, 1993). ICP4 amino acids 316 to 490 are necessary for homodimerization (Everett *et al.*, 1991a; Gallinari *et al.*, 1994) and DNA binding (Everett *et al.*, 1990; Kristie and Roizman, 1986; Wu and Wilcox, 1990), whereas residues 491 to 796 encompass a nuclear localization signal (Paterson and Everett, 1988). ICP4 residues 797 to 1298 are involved in the interaction with TAF250 (Carrozza and DeLuca, 1996; Yao and Schaffer, 1994), and with the HSV-1 IE proteins ICP0 (Yao and Schaffer, 1994), and ICP27 (Panagiotidis *et al.*, 1997), and are required for efficient transcriptional activation (Bruce and Wilcox, 2002; DeLuca and Schaffer, 1988; Paterson and Everett, 1988). ICP4 is highly phosphorylated during infection by at least three cellular kinases, Protein Kinase A (PKA), PKC and cdc2 (Advani *et al.*, 2001; Xia *et al.*, 1996a,b), however the specific role of phosphorylation in regulating the transcriptional activities of ICP4 has not been fully elucidated.

The homologue of ICP4 in VZV is termed IE62 and is encoded by the duplicated genes ORF62 and ORF71, which map to the internal repeats (Piette *et al.*, 1995). IE62 has been shown to induce transcription of all classes of VZV genes, although it has only been recently demonstrated by genetic analysis that IE62 is essential for VZV infection (Sato *et al.*, 2003). IE62 is to a large extent functionally conserved with HSV-1 ICP4, and in fact ICP4 mutants can be complemented by IE62 (Felser *et al.*, 1988). However, there are several important differences. A substantial amount of IE62 is found in the virion tegument (Kinchington *et al.*, 1992), and this appears to require phosphorylation by a VZV encoded protein kinase, ORF 66, which is required for the cytoplasmic localization of IE62 late in infection and the subsequent incorporation of IE62 into virions (Kinchington *et al.*, 2001). Another VZV encoded kinase, ORF 47, which is also a component of the virion, interacts with IE62 and disrupting either the kinase function of ORF47 or its ability to bind to IE62 blocked infectivity of VZV in vivo (Besser *et al.*, 2003). Further, IE62 encodes a potent acidic activation domain in the N-terminus, which is similar to the acidic activation domain of VP16 (Perera *et al.*, 1993). Thus, IE62 also bears some similarity to the HSV-1 transactivator VP16, which is also a component of the virion tegument. Like ICP4, IE62 has been found to interact with other VZV IE proteins. IE62 interacts with IE63 (Lynch *et al.*, 2002), the VZV homologue of HSV-1 ICP22, and with IE4 (Spengler *et al.*, 2000), the homologue of ICP27. The functional importance of these interactions for transcriptional activation of VZV genes during infection remains poorly understood. It has been demonstrated that, in the context of a minimal promoter, a TATA element is sufficient and essential for IE62 activation of transcription, and like its HSV-1 counterpart, IE62 also binds the basal transcription factors TBP and TFIIB in vitro (Perera, 2000). However, upstream binding sites for the cellular transcription factors USF and Sp1 have also been found to be important for the regulation of a number of VZV genes and numerous Sp1 and USF sites have been identified in VZV promoters from all kinetic classes (Ruyechan *et al.*, 2003). USF is a basic-helix-loop-helix-leucine zipper (bHLH-zip) transcription factor that was shown to cooperate with IE62 in the regulation of an early bidirectional promoter driving both the DNA polymerase and DNA-binding protein genes (Meier *et al.*, 1994), and in the regulation of the IE promoter driving IE4 (Michael *et al.*, 1998). Sp1 sites were reported to be important for the regulation of the gE and gI genes (He *et al.*, 2001; Rahaus and Wolff, 2000), and IE62 has been shown to interact with Sp1 in vitro and in vivo (Peng *et al.*, 2003).

Viral IE proteins that contribute to the induced expression of viral genes

In addition to ICP4, HSV-1 encodes three other IE proteins that have regulatory functions. ICP0, a 775 amino acid

nuclear phosphoprotein was first described as a promiscuous transactivator in transient transfection studies because it can transactivate HSV-1 IE, E and L promoters as well as heterologous promoters (for a review, see Everett, 2000). Activation of gene expression by ICP0 was shown to occur at the level of mRNA synthesis (Jordan and Schaffer, 1997). ICP0 does not bind DNA directly (Everett *et al.*, 1991b), and therefore is likely to activate gene expression through interactions with proteins. A large number of interactions have in fact been reported. ICP0 was found to interact with a ubiquitin specific protease, USP7 (Everett *et al.*, 1997), cyclin D3 (Van Sant *et al.*, 1999), elongation factor EF-1δ (Kawaguchi *et al.*, 1997), transcription factor BMAL1 (Kawaguchi *et al.*, 2001), and the HSV-1 transactivator ICP4 (Yao and Schaffer, 1994). ICP0 has also been shown to activate cdk4 and to stabilize cyclins D1 and D3 (Van Sant *et al.*, 2001). However, the mechanism by which ICP0 activates gene expression remains obscure. A major biological activity of ICP0 is the disruption of nuclear structures, termed ND10 or promyelocytic leukemia (PML) bodies. ICP0 encodes two E3 ubiquitin ligase domains, one of which is specified by a RING-finger motif also found in other U3 ubiquitin ligases (Boutell *et al.*, 2002; Hagglund *et al.*, 2002; Hagglund and Roizman, 2002). ICP0 has been linked to the proteasome-dependent degradation of several proteins including the catalytic subunit of DNA protein kinase (Parkinson *et al.*, 1999), the centromere proteins CENP-C and CENP-A (Lomonte *et al.*, 2001), and two components of ND10, PML and Sp100 (Everett *et al.*, 1998a). Because the proteasome inhibitor MG132 interferes with the ability of ICP0 to stimulate viral infection (Everett *et al.*, 1998b), and mutations in the RING finger correlate with ICP0's ability to transactivate (Everett *et al.*, 1991c), it has been thought the destruction of ND10 by ICP0 is an integral event in viral replication. This is supported by evidence from other viruses that also disrupt ND10 structures (reviewed in (Everett, 2001)). However, it has been shown recently that overexpression of PML protein precludes the dispersal of ND10 structures and yet there was no effect on HSV-1 replication (Lopez *et al.*, 2002). Therefore, the mechanism(s) by which ICP0 acts to contribute to virus expression during lytic infection remains to be elucidated.

VZV also encodes a RING-finger protein termed IE61. The N-terminal RING finger domain is the only obvious similarity to ICP0. IE61 does not contain a USP7 binding domain and does not affect the distribution of USP7 (Parkinson and Everett, 2000). IE61 does induce colocalizing conjugated ubiquitin, suggesting that it may also act as an ubiquitin ligase (Parkinson and Everett, 2001). Little else has been reported on the activity of VZV IE 61.

Another HSV-1 IE protein that has been implicated in the regulation of viral IE and L gene expression is ICP22. ICP22 is not essential for virus infection in cultured cells, and mutants grow as well as wild-type virus in Vero and HEp-2 cells, but ICP22 mutants do exhibit a host range and replicate much less efficiently in primary human, rabbit and rodent cells. In these latter cells the expression of a subset of late genes is greatly reduced (Ogle and Roizman, 1999). Further, phosphorylation of ICP22 by an HSV-1 encoded kinase, UL13, is required for the accumulation of a subset of both IE and L genes in primary human, rodent and rabbit cells (Purves *et al.*, 1993) and this appears to be mediated by the activation of the cdc2 cyclin-dependent kinase (Advani *et al.*, 2000). A possible mechanism for the requirement for ICP22 and UL13 for appropriate expression of L transcripts is the intriguing finding that ICP22 and UL13 are both required for an HSV-1 induced modification of RNA polymerase II (Long *et al.*, 1999). Specifically, infection with HSV-1 results in the depletion of both the hypo- and hyperphosphorylated forms of RNA polymerase II, known as the IIa and IIo forms, which represent the initiating and elongating forms of RNA polymerase II. Instead, early in HSV-1 infection, an intermediately phosphorylated form appears (Rice *et al.*, 1995). Infection of human primary embryonic lungs cells with mutants defective in ICP22 or UL13 led to significantly reduced viral gene transcription at late times after infection (Long *et al.*, 1999). These results suggest that the modification in the phosphorylation of RNA polymerase by ICP22 and UL13 promotes HSV-1 transcription over transcription of cellular genes. However, despite the attractiveness of this model, there are findings that cannot be explained solely by the modification of the phosphorylation of RNA polymerase II. First, ICP22 is not required for L gene expression in many cell lines, and the modification of RNA polymerase II to the intermediate form is not seen in infections with ICP22 or UL13 mutants. Thus, this modification is not absolutely required for transcription of HSV-1 genes. Second, RNA polymerase II is still recruited to viral transcription/replication compartments, whether or not it is modified, thus altered phosphorylation is not a prerequisite for its nuclear relocalization (Rice *et al.*, 1995). Third, an additional change to the RNA polymerase II holoenzyme was reported that depends on HSV-1 IE proteins, but not specifically on ICP22. That is, the loss of the general transcription factor TFIIE from the holoenzyme was found to require HSV-1 IE proteins, which may be redundant because no single IE deletion mutant eliminated this change (Jenkins and Spencer, 2001). TFIIE is necessary for activated transcription initiation in uninfected cells, and thus the loss of this general transcription factor from the complex may result in the repression of

transcription of cellular genes that has been reported following HSV-1 infection (Spencer *et al.*, 1997).

It has been shown recently that the phosphoserine-2 form of the RNA polymerase C-terminal terminal domain, which is found in elongating transcription complexes, is decreased during HSV-1 infection (Fraser and Rice, 2005; Dai-Ju *et al.*, 2006). Furthermore, total levels of RNA polymerase were decreased indicating that this was due to a loss of protein, indicating protein degradation rather than dephosphorylation, and proteasome inhibitors prevented the degradation (Dai-Ju *et al.*, 2006). The degradation appeared to be due to robust HSV-1 transcription because in infections with viral mutants in which viral transcription was greatly reduced, or in which viral transcription was blocked by inhibitors, degradation was prevented. By contrast preventing the degradation of the elongating serine-2 phosphorylated RNA polymerase II decreased late viral gene expression and viral yields (Dai-Ju *et al.*, 2006). These findings suggest that during periods of robust viral transcription in HSV-1 infected cells, elongating polymerase complexes may become arrested or stalled because of a pile-up of transcribing complexes on families of colinear transcripts, or because of collisions of transcribing complexes on opposite strands that are being transcribed at the same time. Resolution of these stalled complexes by proteasomal degradation may thus be required to allow transcription to resume and proceed through the gene.

Interestingly, the VZV homologue of ICP22, termed IE63 has been shown to be essential for VZV infection (Sommer *et al.*, 2001), although it does not appear to play a major role in gene regulation on its own. IE63 was reported to be able to repress two VZV promoters in an activity assay and this repression was regulated by phosphorylation by two cellular casein kinases (Bontems *et al.*, 2002b). However, in another report, IE63 was found to have no transcriptional activating or repressing activity within the context of a minimal VZV glycoprotein promoter, however, the presence of IE63 upregulated IE62 transactivation of the promoter (Lynch *et al.*, 2002). Further, IE63 protein was shown to interact with IE62 through the N-terminal 142 amino acids, and a portion of the IE63 and IE62 proteins colocalized in VZV-infected cells (Lynch *et al.*, 2002). IE63 is phosphorylated in infected cells and can be phosphorylated in vitro by casein kinase II (Bontems *et al.*, 2002a; Stevenson *et al.*, 1996). Further, IE63 has been found to be heavily phosphorylated by, and tightly bound to, the VZV ORF47 kinase (Kenyon *et al.*, 2003), a homologue of HSV-1 UL13, which phosphorylates ICP22. Intriguingly, IE63 protein can be coimmunoprecipitated with the cellular RNA polymerase II from infected cell extracts, indicating that it is present in a complex with that enzyme (Lynch *et al.*, 2002). It has not been demonstrated whether or not the IE63-ORF47 complex can alter the phosphorylation of RNA polymerase II in VZV-infected cells. See Fig. 9.1.

RNA processing and transport

Following synthesis of transcripts in eukaryotic cells, the nascent pre-mRNAs are processed by capping at the 5′ end, cleavage and polyadenylation to form the 3′ end, and splicing to remove intervening sequences. After processing, mRNAs must be exported through the nuclear pore complex to the cytoplasm for translation. Export of mRNAs requires binding by RNA export adaptor proteins, recognition by export factors and translocation through the nuclear pore complex (NPC). Three classes of factors appear to be required for mRNA export: adaptor proteins that bind directly to the mRNA, receptor proteins that recognize and bind to adaptor proteins, and nuclear pore complex components termed nucleoporins that mediate export across the nuclear membrane (for review see (Komeili and O'Shea, 2001; Zenklusen and Stutz, 2001). In metazoan cells, the processing events occur co-transcriptionally because the processing factors are recruited to the sites on the nascent transcripts where their activities are required by binding to the highly conserved C-terminal domain (CTD) of RNA polymerase II (for review see Reed, 2003). In addition, RNA export is closely coupled to splicing (Luo and Reed, 1999). This occurs because a complex of proteins that includes one or more export adaptor proteins binds to the pre-mRNA at a specific site just upstream of what will become exon–exon junctions in the spliced product. This so-called exon junction complex or EJC remains bound to the spliced RNA and escorts the mRNA to the cellular mRNA export receptor, termed TAP or NXF1, with which the export adaptor proteins interact directly. The mRNP complex is then exported through the nuclear pore (for review see Reed and Magni, 2001).

In HSV-1 infected cells, viral transcripts are processed by 5′ capping and 3′ end formation by the cellular machinery. However, it has been reported that some polyadenylation sites present primarily on late transcripts are utilized inefficiently by cellular polyadenylation factors and these sites were operationally defined as weak. The activity of an HSV-1 IE protein, ICP27 stimulated the use of these sites in vitro (McLauchlan *et al.*, 1992). Further analysis suggested that ICP27 recruits the cleavage stimulation factor, Cst64, which is required for poly(A) site recognition, to weak HSV-1 poly(A) sites (McGregor *et al.*, 1996). However, a study investigating differential polyadenylation of two transcripts that arise from the UL24 gene by the use

of two different poly(A) sites, one weak and one strong, found that the accumulation of transcripts during infection from the weak poly(A) site did not require ICP27 (Hann *et al.*, 1998). In addition, a recent study using various HSV-1 gC gene plasmid constructs showed that the stimulatory effect of ICP27 occurred post-transcriptionally but was independent of the inserted polyadenylation site (Perkins *et al.*, 2003). Thus, the role of ICP27 in enhancing the use of weak poly(A) sites has not been firmly established.

The role of ICP27 in another RNA processing event has been defined to a significant extent, namely the effect of ICP27 on pre-mRNA splicing. Although most metazoan mRNAs are multiply spliced, the majority of HSV-1 transcripts are intronless. This presents an interesting paradox for the virus because cellular RNA export is intimately coupled to splicing through the deposition of the exon junction complex on the spliced mRNAs, which marks mRNAs for export by TAP/NXF1 (Cullen, 2000). Although there are cellular transcripts that are intronless, these transcripts have been found to contain specific recognition elements that allows their export via TAP/NXF1 (Huang *et al.*, 2003). Following infection with HSV-1, the cell is presented with thousands of viral intronless transcripts that would compete poorly with cellular transcripts for export. ICP27 shifts the balance by shutting down the cellular splicing machinery, albeit temporarily, thus cellular pre-mRNAs are not fully processed and exported (Bryant *et al.*, 2001; Sciabica *et al.*, 2003). Further, ICP27 recruits an export adaptor protein, termed Aly/REF, which is part of the EJC, to sites of HSV-1 transcription (Chen *et al.*, 2002). Thus, HSV-1 mRNAs are given access to the TAP/NXF1 pathway (Chen *et al.*, 2002; Koffa *et al.*, 2001). ICP27 inhibits cellular splicing at early times after infection by recruiting a predominantly cellular kinase, SRPK1, to the nucleus of infected cells where it inappropriately phosphorylates a family of splicing factors, termed SR proteins, which are required for splicesome assembly (Sciabica *et al.*, 2003). Proper phosphorylation is required for SR proteins to perform their roles in splicing and consequently, splicesome complex formation is stalled. ICP27 interacts with SR proteins and other spliceosomal components (Bryant *et al.*, 2001; Sciabica *et al.*, 2003), and most likely then encounters Aly/REF, the export adaptor, which is part of the EJC (Chen *et al.*, 2002; Koffa *et al.*, 2001). ICP27 recruits Aly/REF to sites of HSV-1 transcription, marked by staining with an antibody to ICP4 (Chen *et al.*, 2005), and ICP27 then binds to HSV-1 intronless transcripts through its RGG box RNA binding motif (Sandri-Goldin, 1998). The RNA-ICP27-Aly/REF complex binds to TAP/NFX1 through the interaction of both ICP27 and Aly/REF with TAP/NXF1 (Chen *et al.*, 2002), and HSV-1 transcripts are exported through the NPC. Splicing is restored at later times of infection, when ICP27 is actively shuttling and exporting viral mRNA. The nature of the switch between these early and late activities of ICP27 has not been defined but could involve post-translational modifications. ICP27 is phosphorylated by several cellular kinases (Zhi and Sandri-Goldin, 1999) and it undergoes arginine-methylation in the RGG box (Mears and Rice, 1996). A recent report suggests that ICP27 may be directed initially to sites of cellular transcription and splicing, similar to cellular RNA processing factors, by interacting with RNA polymerase II. ICP27 was found to co-immunoprecipitate with RNA polymerase II in HSV-1-infected cells (Zhou and Knipe, 2001). Further, ICP27 was found to interact directly with the CTD of RNA polymerase II in vitro and in virus-infected cells (Dai-Ju *et al.*, 2006).

The homologue of ICP27 in VZV is IE4. The IE4 protein is a transactivator of gene expression whose regulatory properties are not fully understood. IE4 stimulates VZV gene expression and it also is capable of heterologous transactivation (Defechereux *et al.*, 1997; Perera *et al.*, 1994), and IE4 has been suggested to exert its function through both transcriptional and post-transcriptional mechanisms (Defechereux *et al.*, 1993; Defechereux *et al.*, 1997; Perera *et al.*, 1994). IE4 shares considerable amino acid sequence homology with HSV-1 ICP27, especially in the carboxyl terminus and in the central part of the protein (Davison and Scott, 1986). The carboxyl-terminal region of ICP27 that is rich in cysteine and histidine residues has been shown to bind zinc (Vaughan *et al.*, 1992) and to be required for multimerization (Zhi *et al.*, 1999). Whereas the carboxyl-terminal region of IE4 also contains cysteine and histidine residues, it is not known whether this region binds zinc, and dimerization of IE4 was found to require the C-terminal cysteine-rich domain and the central region of the protein (Baudoux *et al.*, 2000). The amino-terminal regions of the two proteins have a more limited amino acid homology; however, both are highly acidic. In transfection assays, it was demonstrated that the IE4 N-terminal acidic region was required for its trans-activating function and that it could be replaced in part by substituting the corresponding region of ICP27 (Moriuchi *et al.*, 1995). While IE4 is not a potent transactivator on its own, it can act as a major coactivator of transactivation mediated by IE62, and was found to interact with IE62 in infected cells and in vitro (Spengler *et al.*, 2000). The interaction depended on the phosphorylation state of IE62. The nature of the post-transcriptional activation seen with IE4 has not been defined, and it is not known if IE4 is involved in the regulation of splicing or viral RNA export. Many VZV transcripts are also intronless, and IE4 was reported to shuttle between the nucleus and

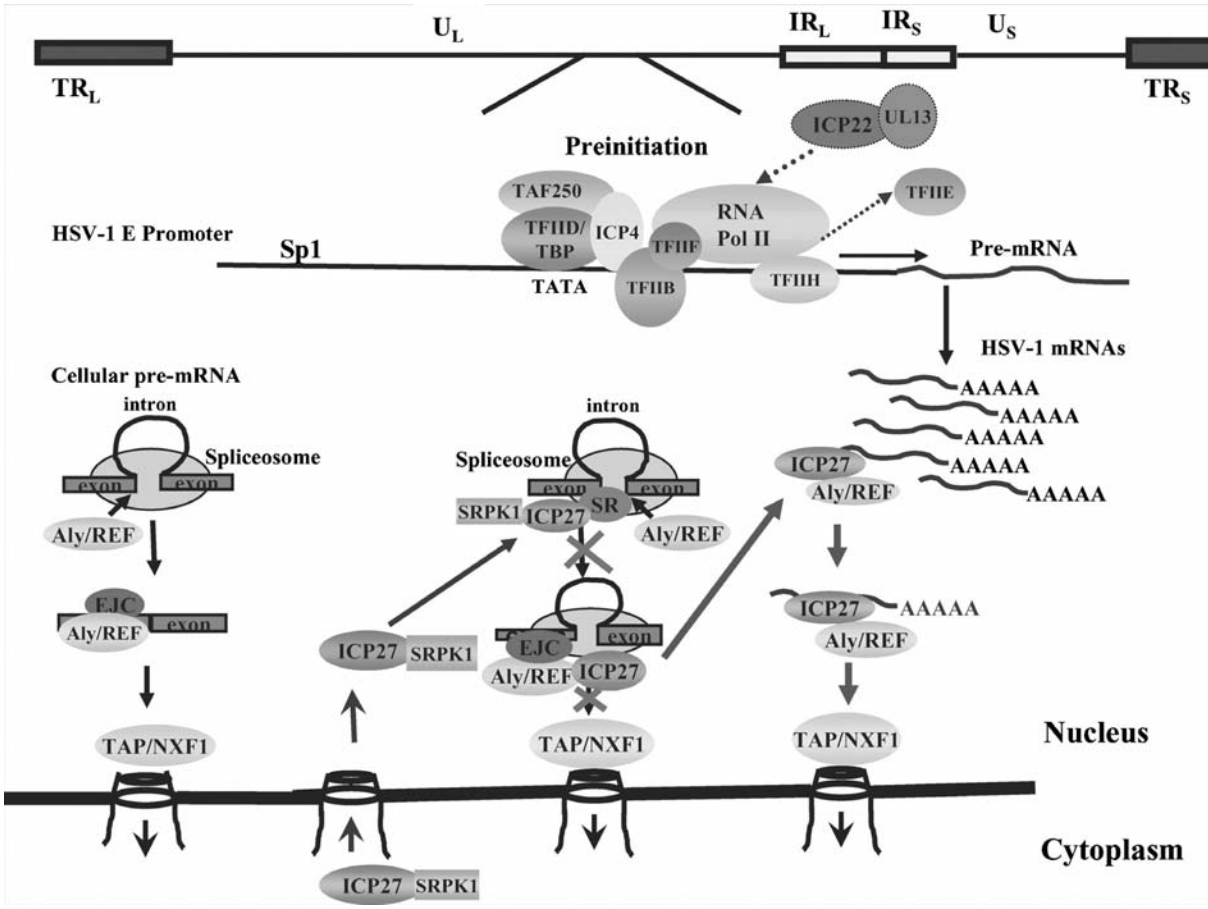

Fig. 9.1. Model of HSV-1 early (E) gene expression showing the initiation of transcription, RNA synthesis and export. The HSV-1 genome is depicted in schematic form. An E gene promoter from the U_L region is blown up to illustrate the upstream Sp1 site and the TATA box. The HSV-1 modified preinitiation complex includes ICP4, which forms a tripartite complex with TBP, a component of TFIID, and TFIIB (Grondin and DeLuca, 2000). ICP4 also interacts with TAF250, which stabilizes the interaction of TFIID with DNA (Carrozza and DeLuca, 1996). In VZV infected cells, IE62 has also been shown to bind TBP and TFIIB (Perera, 2000). The altered phosphorylation of RNA polymerase II by ICP22 and U_L 13 (Long *et al.*, 1999) is shown by a dashed line, as is the release of TFIIE from RNA polymerase holoenzyme (Jenkins and Spencer, 2001). The pathway for mRNA export in uninfected cells is disrupted during HSV-1 infection. ICP27 recruits SRPK1 to the nucleus where it inappropriately phosphorylates the splicing SR proteins and splicesome assembly is stalled (Sciabica *et al.*, 2003). ICP27 encounters the export adaptor Aly/REF, which is part of the EJC and directs it to HSV-1 intronless transcripts (Chen *et al.*, 2002; Koffa *et al.*, 2001). ICP27 binds viral intronless RNA and the ICP27-RNA-Aly/Ref complex interacts with the export receptor TAP/NXF1 to export viral RNAs to the cytoplasm. It has not been demonstrated whether VZV IE4 affects splicing or is involved in viral RNA export.

cytoplasm, however, this activity was not further delineated (Baudoux *et al.*, 2000). See Fig. 9.1.

Concluding remarks

Both HSV-1 and VZV have evolved mechanisms for usurping the cellular machinery to produce viral transcripts to insure the successful generation of viral progeny. The viral regulatory proteins that are involved in the initiation of transcription, RNA processing and export are the IE proteins in both viruses. These proteins all have unique roles in the expression of viral gene products. While the HSV-1 and VZV IE proteins share homology, they have clearly diverged to meet the unique requirements of their parent viruses. For example, HSV-1 IE proteins are not significant components of the virion tegument, whereas, VZV IE62, IE4 and IE63 are. Further, HSV-1 IE proteins are not expressed in latently infected cells; however, VZV IE63 is expressed as a major

protein during latency along with IE4 (Kenyon *et al.*, 2003). Both HSV-1 and VZV encode essential IE proteins, ICP4 and IE62, that transactivate viral gene expression, and both encode IE proteins, ICP22 and IE63, that interact with or modify RNA polymerase II, although the functional repercussions of this are not yet fully appreciated. The roles of the RING finger proteins, ICP0 and IE61 have not been fully elucidated despite the fact that we now know a great about what these proteins do in the infected cell. While HSV-1 hijacks the cellular export machinery via ICP27 to facilitate translation of its transcripts, little is known about how the switch from an early splicing inhibitor to a late viral export factor is controlled. It remains to be seen if VZV IE4 functions in this manner. Thus, there is still much to learn about gene regulation in the alphaherpesviruses.

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10

Alphaherpesvirus DNA replication

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DNA replication in alphaherpesviruses has been the subject of study in bursts over the years. Interest in the subject depends not just on simple curiosity about this central feature of the viral growth cycle, but also because DNA replication is a potentially useful target for antiviral therapy, as has already been shown with agents such as acyclovir. The viral contributions to the mechanism of genome replication are quite well understood but we still are unable to duplicate the in vivo situation in an in vitro assay. Much of the recent interesting work involves the host cell's contribution to the process, and this seems likely to remain a focus for the future.

Structure of the genome

There are over 30 alphaherpesviruses that infect a wide range of host species. Their genomes fall into two general categories, either herpes simplex (HSV) – like or varicella zoster (VZV) – like, with four or two, respectively, isomeric forms (Fig. 10.1). There is a wide range of G + C content (32%–75%), with a bias towards higher (>50%) numbers. There is also size heterogeneity (125–180 kbp) which, although quantitatively less than the nucleotide composition variation, may be much more significant for the lifestyle of the virus. All alphaherpesvirus genomes contain four general structural components: unique long and short (U_L, U_S) sequences that encode single-copy genes and inverted repeat regions that bound the unique regions; these may contain diploid genes and sequences required for cleavage and packaging of viral DNA (Fig. 10.1). DNA replication initiates at origin sequences (*ori*), and there are two or three of these in each genome, depending on the virus. Evidence suggests that only one *ori* is required for viral DNA replication, however, and the importance of multiple origins, if any, remains to be discovered (Roizman and Knipe, 2001; Balliet *et al.*, 2005). Only a few of the alphaherpesviruses have been studied at the molecular level regarding DNA replication (e.g., HSV, VZV, equine herpesvirus type 1 (EHV-1), pseudorabies virus (SuHV-1), and infectious bovine rhinotracheitis virus (BoHV-1). Most of the work, however, has been carried out with HSV, reviewed by Boehmer and Lehman (1997) and Lehman and Boehmer, (1999); the majority of the information presented here will be that gathered for HSV.

The origins of DNA replication

For those viruses with an ori_L, this is found in the middle of the U_L segment, in the region lying between DNA polymerase and the major DNA-binding protein for HSV, but not necessarily for other viruses (Telford *et al.*, 1992). The ori_S sequences sit in the repeat regions bounding the

$a_1\ a_n\ b$---------- ori_L ---------- $b'\ a_n'\ c'\ ori_S$ -------- $ori_S\ c\ a_S$

$<TR_L><\qquad U_L \qquad>< IR_L\ IR_S>< U_S><TR_S>$

Fig. 10.1. The structure of alphaherpesvirus genomes. The upper diagram shows the sequence organization of the herpes simplex virus genome, with the origins of DNA replication shown as ori_L or ori_S. The lower diagram shows the general layout of the regions of the genome, with TR = terminal repeat, IR = internal repeat and the subscripts $_L$ and $_S$ denoting long and short regions, respectively. This is the scheme for the simplexviruses, that invert the U_L and U_S regions relative to each other, to yield four isomeric forms of the genome. For the varicelloviruses, the TR_L and TR_S sequences are so short that U_L does not invert, and there are only two isomeric genome forms, resulting from inversion of U_S. In addition, the varicelloviruses have no ori_L but do have two copies of ori_S.

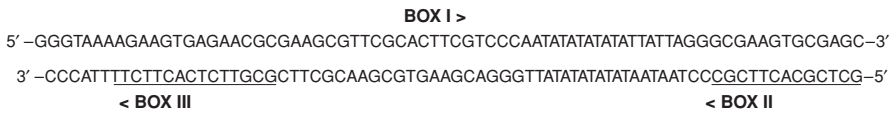

Fig. 10.2. The structure of alphaherpesvirus origins of replication. Shown above is the HSV-1 ori_S sequence. Box III is a low-affinity UL9 recognition site, Box I is a high affinity UL9 binding site and Box II has a ten-fold lower affinity for UL9 than Box I. The ori_S has a large dyad symmetry element (45 bp) centered around an AT region of 18 bp. The ori_L is similar but has a larger symmetry element (144 bp) and AT region (20 bp); in addition, it has a second copy of Box III and, instead of Box II, has a second copy of Box I. Other viral origins have analagous structural elements.

U_S region. The two origins in HSV are very similar but not identical (Fig. 10.2), and other alphaherpesvirus origins also have the same general structural features (Telford *et al.*, 1992; Kuperschmidt *et al.*, 1991), to the extent that HSV infection can support limited replication of a VZV *ori*-containing plasmid (Stow and Davison, 1986), for example. These herpesvirus origins are not dissimilar to other origins in other mammalian viruses, with a dyad symmetry element adjacent to an AT-rich sequence, flanked by binding sites for the origin recognition protein and possibly other factors (Fig. 10.2), implying similar general mechanisms for initiation of genome replication.

Location of DNA synthesis

Viral DNA replication takes place in the nucleus. Prereplicative sites form as DNA-protein complexes that appear as punctuate elements by immunofluorescence microscopy, in association with nuclear domain 10 (ND10) (Ishov and Maul, 1996). As synthesis proceeds, larger areas of the nucleus become involved, and are visualized as globular replication compartments (Quinlan *et al.*, 1984). Recently, it has been demonstrated that active viral transcription assists in the association of viral DNA with ND10 (Sourvinos and Everett, 2002), implying that assembly of transcription and/or replication complexes promote this association. The authors suggest that a critical step in initiating DNA synthesis is ND10 association, and this idea is supported by work with Epstein–Barr virus, in which postreactivation, but not latent, genomes are ND10-associated (Bell *et al.*, 2000).

Proteins involved in DNA synthesis

It is generally accepted that, prior to replication, the linear viral DNA initially circularizes; then synthesis initiates at an origin(s) and proceeds bidirectionally to form theta structures. Soon after, synthesis switches to a rolling circle mode, in which genome-sized pieces are cleaved and packaged as they are produced. This all takes place in the presence of UL9, the origin-binding protein; UL29 (ICP8), a single-strand DNA-binding protein; UL30 and UL42, the DNA polymerase and DNA polymerase processivity factor, which make up the DNA polymerase complex; and UL5, 52 and 8 which make up the DNA helicase/primase complex. These constitute, at present, the essential alphaherpesvirus replication proteins. The assembly of the viral replication complex appears to be an ordered process, in which UL9, ICP8 and the helicase-primase heterotrimer first form replication foci, and the polymerase holoenzyme (UL30 and UL42) are then recruited; this recruitment process seems to require primer synthesis (Carrington-Lawrence and Weller, 2003). Despite our knowledge of the viral polypeptides required for origin-dependent DNA synthesis in vivo, it has still not been possible to reconstitute this theta mode of replication in vitro. Instead, work has focused on rolling circle replication, which can be demonstrated using a replisome containing ori_S and the two viral protein complexes – DNA polymerase/UL42 and the heterotrimeric helicase-primase; the viral SSB (UL29) is not required (Falkenberg *et al.*, 2000).

The origin-binding protein dimerizes and binds to the CGTTCGCACTT *ori* sequence; it also has ATP-binding and helicase functions. Opening the viral *ori* appears to involve several viral and cellular proteins; on a superhelical template in the presence of ATP, UL9 and ICP8 allowed about half of the *ori* s to unwind, and following addition of the cellular topoisomerase I, the extent of unwinding was augmented to >1 kb.

ICP8, the UL29 gene product, is a classical single-stranded DNA binding protein (SSB), with helix-destabilizing activity. It has been shown to interact with all the other replication proteins (above) and seems likely to be the principal scaffold for the generation of prereplication complexes adjacent to ND10 sites. Recent work shows that a C-terminal alpha helix is important in binding viral and/or cellular factors, allowing targeting of ICP8 to specific nuclear sites (Taylor and Knipe, 2003). In addition, it seems to operate in concert with the alkaline endo-exonuclease activity of the virus to constitute a viral recombinase activity similar to that of the bacteriophage lambda (Reuven *et al.*, 2003). In that context, it has recently

been shown to stimulate, and regulate, the processivity of, the pseudorabies virus DNase (Hsiang, 2002).

DNA polymerase is a heterodimer, with the UL30 gene product as the typical polymerase/3′–5′ exonuclease (proofreading) activities, and UL42 as the processivity factor. The holoenzyme has a broad substrate specificity that allows it to be a useful target for antiviral therapy, as discussed elsewhere in this volume. The UL42 protein stimulates polymerase activity and increases the fidelity of replication (Chaudhuri *et al.*, 2003); it has been hypothesized to function by interdigitation of its termini, using a hinge region at aa241–261 (Thornton *et al.*, 2000). C-terminal residues in the polymerase polypeptide are important for UL42 interaction and interference with this may be a useful antiviral tool (Bridges *et al.*, 2000). UL42 is an unusual processivity factor, in that it binds directly to DNA, unlike the "sliding clamps" such as PCNA and the *E. coli* β protein. Nevertheless, it seems to resemble PCNA both in its interaction with polymerase (Zuccola *et al.*, 2000) and its ability to slide downstream with polymerase during replication (Randell and Coen, 2001).

The UL5 and UL52 polypeptides constitute the core of the helicase–primase complex, with DNA helicase, primase and ATPase activities. UL5 and UL52 also demonstrate DNA-binding activity when they form complexes, and this DNA-binding activity is preferentially to forked substrates, as opposed to single-stranded or duplex molecules (Biswas and Weller, 2001). The contribution of UL8 to the complex is to work with ICP8 to promote unwinding activity and to catalyze nuclear localization of the complex. An HSV-1/HSV-2 recombinant, in which UL5 is the only HSV-2 gene, is non-neurovirulent and defective in DNA replication in neurons. The primary defect was in primase activity, suggesting that interactions between subunits in the complex are vital in ensuring its full catalytic activity (Barrera *et al.*, 1998). Inhibitors of helicase–primase activity have recently been investigated as potential antiviral agents. The most powerful compounds inhibited primase, helicase and ATPase activities and, as expected, were active against viral mutants resistant to nucleoside-based therapies (Crute *et al.*, 2002).

A second set of proteins with links to DNA synthesis are considered non-essential for replication in cultured cells, but several appear to be essential for "normal" behavior of virus in animal models. These include: pyrimidine deoxynucleoside kinase; alkaline endo-exonuclease; ribonucleotide reductase; uracil N-glycosylase and deoyuridine triphosphatase.

The pyrimidine deoxynucleoside kinase, popularly known as thymidine kinase (TK) phosphorylates a wide range of nucleoside substrates, as well as TMP, and is responsible for the rise in the TTP pool that is characteristic of HSV-infected cells. The enzyme, because of its broad specificity, also acts on the acyclovir family of antivirals, and has been used extensively in gene therapy in combination with ganciclovir and acyclovir (Hayashi *et al.*, 2002). This broad specificity has recently been analysed and seems to depend on the electric dipole moment of ligands interacting with a negatively charged residue at aa 225 (Glu) (Sulpizi *et al.*, 2001). TK-deletion mutants will establish latency in mouse ganglia but do not reactivate, presumably owing to a lack of an equivalent cellular activity. However, viral strains that only produce small quantities of enzyme are able to reactivate with wild-type efficiency (Griffiths *et al.*, 2003).

The UL12 ORF encodes an endo-exonuclease that is most active between pH 9 and 10; the significance of this is uncertain. This protein interacts with ICP8 and plays a role in the maturation and packaging of viral DNA; this is consistent with the behavior of UL12 null mutants, which make DNA and late proteins, but do not produce infectious virus particles efficiently. The hypothesis is that the enzyme acts on gaps and/or nicks in progeny DNA, either to process or repair it on its way towards encapsidation. The UL12 phosphoprotein and ICP8 can also work in concert to promote strand exchange, similar to recombination events in lambda phage (Reuven *et al.*, 2003).

Ribonucleotide reductase allows formation of deoxynucleoside diphosphates from ribonucleoside substrates and is not negatively regulated by the high TTP pools in HSV-infected cells, as would be the cellular equivalent. It is made up from the products of two ORFs (UL39, R1 and UL40, R2) to form a symmetric heterotetramer, and seems to be necessary for viral growth in "resting" cells. There are reports of protein kinase activity associated with the R1 polypeptide of HSV-2, but there is also evidence that, while HSV-2 R1 is itself a substrate for protein kinase activity, the polypeptide does not itself possess intrinsic protein kinase activity (Langelier *et al.*, 1998). Recently it has been shown that an accessory function of the reductase may be to protect HSV-1 infected cells against cytokine-induced apoptosis.

Uracil N-glycosylase (encoded in UL2) is a repair enzyme that cleaves mutated U residues from the DNA sugar backbone resulting from a cytosine deamination event, subsequent to repair by viral and cellular enzymes. It is curious that the virus should encode an enzyme that is ubiquitous in host cells, but activities may be low in non-dividing cells that the virus encounters in vivo. This may also be the explanation for reduction in neurovirulence and a poor ability to reactivate from latency that is characteristic of UL2-deletion mutants.

Deoxyuridine triphosphatase (dUTPase), the product of the UL50 gene, breaks down dUTP, preventing dU incorporation into viral DNA. At the same time, the product of its action, dUMP, is a substrate for the pathway that leads

to TTP synthesis. UL50 null mutants are impaired in their ability to replicate in the central nervous system in mice, although they seem capable of normal growth in peripheral tissues. They also fail to reactivate effectively.

In addition to the viral proteins described above, there are cellular proteins that contribute both essential and/or accessory roles in viral DNA replication. Among the obvious examples of the former are DNA ligase and topoisomerase activities, as well as repair endonuclease activity and an endonuclease G, that may contribute to maturation of the viral genome (Huang *et al.*, 2002). Among the latter are the numerous cellular enzymes that contribute substrates for DNA synthesis, and that are found in all dividing cells. There will likely be additional cellular proteins found, however, such as the promyelocytic leukemia protein (PML) that is recruited to replication foci following the arrival of the polymerase complex (Carrington-Lawrence *et al.*, 2003), and the OF-1 protein that has a function in initiation of replication (Baker *et al.*, 2000). It also contains the Ku70/Ku80 heterodimer which is present in origin-specific DNA-binding complexes in primates and yeast (Murata *et al.*, 2004).

DNA replication and the cell cycle

HSV downregulates host cell DNA synthesis during lytic infection, implying that the normal cell cycle is dysregulated by the virus. The reason for this, presumably, is to allow the virus maximum access to DNA precursors, replication sites and replication proteins that would otherwise be involved in cellular genome synthesis. In a parallel scenario, it has been shown that, using the inhibitor roscovitine, cyclin-dependent kinases are required for HSV DNA replication, even although the early viral proteins required for synthesis are all present (Schang *et al.*, 2000). Cell cycle arrest has been shown to involve the viral ICP0 protein, using mechanisms that appear to be both p53-dependent and p53-independent. One mechanism whereby ICP0 is effective, is through arresting the cycle at the G1->S stage, blocking cells at the pseudo-prometaphase stage of mitosis (Lomonte and Everett, 1999). The data suggest that viral factors other than ICP0 may be involved in this cell cycle block but that ICP0 alone may be responsible for the mitotic block. The authors make the point that ICP0 expression is incompatible with the growth of a cell population. One additional viral protein that affects the cell cycle is ICP27; it blocks the cycle at S phase, through inhibiting the phosphorylation of pRb (Song *et al.*, 2001). Thus, the virus has evolved a number of different mechanisms to interfere with normal cell cycle progression, emphasizing the potential importance of this step in the viral growth cycle.

Maturation and packaging of viral DNA

The a sequences of the HSV genome (Fig. 10.1) constitute the *cis*-acting signals for cleavage of the newly synthesized DNA, resulting from rolling circle replication, into genome-sized pieces for packaging into capsids. The DR1 repeat sequences that flank the a sequences contain the actual cleavage site, and two unique regions, Uc and Ub, are next to the genomic ends. A fragment, Uc/DR1/Ub, is sufficient to constitute a minimal packaging signal, and elements inside the U sequences can be found in other herpesvirus genomes; these are the pac elements. Data suggest that these pac sequences contain signals for both initiation and termination of packaging (White *et al.*, 2003). The proteins involved in the process are UL6, UL15 and UL28 in HSV-1, and these are sufficient for cleavage of the concatameric DNA, as well as their packaging into procapsids. UL6 likely constitutes the gateway on the preformed capsid for entry of the genome and is present at one vertex, while a UL15/UL28 complex has the properties of the engine that drives the genome into the procapsid. UL6 is capable of specific interaction with both UL15 and UL28 (Hodge and Stow, 2001). A fourth protein, UL25, seems to play a final role in the packaging process, prior to the movement of nucleocapsids into the cytoplasm.

Recombination

Homologous recombination is a frequent event in herpesvirus infected cells, and it has been used experimentally to investigate gene functions, for example, by generation of HSV-1/HSV-2 intertypic recombinants. Viral DNA molecules that are undergoing replication are the best substrates for recombination and the genomic inversions that give rise to the different isoforms of the HSV genome are generated through recombination involving the repeat regions. While it seems likely that cellular enzymes or other proteins will be important in the process, there are clearly roles for viral gene products. For example, ICP8, as outlined earlier, appears potentially to be a key player in the recombination process. In in vitro assays, it promotes strand exchange in conjunction with the viral helicase-primase and catalyzes single-strand invasion in an ATP-independent manner (Nimonkar and Boehmer, 2003). It also may be involved in single-strand transfer, in collaboration with the HSV endo-exonuclease activity, UL12. The 5′–3′ exonuclease activity of this protein shares homology with the lambda exonuclease (Redalpha) that is known to be essential for homologous recombination in the phage. It is proposed that the two viral proteins work in concert to

promote strand exchange, shown experimentally by generating a gapped circle and a displaced strand from an M13 duplex DNA molecule and an M13 single-stranded circular DNA molecule. Interestingly, UL12 polypeptide that lacked nuclease activity was incapable of catalyzing this experimental recombination (Reuven *et al.*, 2003). In addition to the various roles that recombination might play in the virus life cycle, it has been proposed that recombination may also be important for the switch that occurs between the early theta mode of replication of viral DNA and the mainstream rolling circle mode, in a way reminiscent of the mechanisms occurring in the lytic phase of lambda replication (Boehmer and Lehman, 1997).

Latency

Alphaherpesvirus latency, as typified by HSV, is characterized by a lack of infectious virus in latently infected neural tissue and expression of specific LATs (latency-associated transcripts). VZV, on the other hand, has no LATs, and expresses a restricted set of transcripts and proteins seen in the early phases of normal lytic infection. Calculations of the numbers of viral genomes present in latently-infected neurons have shown a small but significant number to be present; in HSV, a recent estimate gives a mean of 178 per LAT-positive neuron using laser capture microdissection (Chen *et al.,* 2002). This number is not dramatically different from less sophisticated measurements on VZV-infected ganglia. This raises the issue of how this number of genomes arises, and the simplest explanation is that initial infection of the neuron proceeds by the normal lytic route, initiates some DNA replication (only through a theta-like mode?) but is quickly curtailed by factors within the cell. At present, we have no clues as to what constitutes this inhibitory mechanism. Reactivation must involve renewed viral DNA synthesis and, indeed, the presence of several viral proteins of the "non-essential-in-cell-culture" variety is necessary to allow the process to proceed. In addition, there is a proposal that the cellular C1 factor, responsible for assembly of transcriptional enhancer complexes, is normally missing from neuronal nuclei but present soon after reactivation (Kristie *et al.,* 1999). Thus, as with other herpesvirus systems (e.g., EBV), it is likely that the HSV reactivation is initiated through production of gene regulatory viral proteins.

Future directions

Future investigation is likely increasingly to focus on the host cell's contribution to viral genome replication. The recent interest in nuclear structures will provide the field with a new set of findings that may provide the clues necessary to allow faithful replication of the in vivo situation in an in vitro environment. Aside from replication, the host cell also plays a role in the repair/recombination events that characterize the viral growth cycle, and these contributions also remain to be precisely defined. Finally, the issue of latent vs. lytic viral behavior has focused primarily on transcription and its control; perhaps it is now time to look more closely at viral DNA replication in latently infected cells.

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11

Envelopment of herpes simplex virus nucleocapsids at the inner nuclear membrane

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Introduction

As in all herpesviruses, Herpes simplex nucleocapsids assembled in the nucleoplasm obtain an initial envelope by budding through the inner nuclear membrane of infected cells. This chapter will focus on the proteins responsible for nucleocapsid budding in the herpes simplex virus system. Of interest is the observation that orthologs of at least the UL31 and UL34 genes of herpes simplex virus genes likely mediate similar functions in members of both the beta- and gamma herpesvirinae (Muranyi *et al.*, 2002; Gonnella *et al.*, 2005). Thus, it is expected that this information will be relevant to the study of nucleocapsid envelopment of all herpesviruses.

Anatomy of the nuclear membrane: it's all connected

The nuclear envelope consists of two leaflets: the inner leaflet or inner nuclear membrane (INM) partitions the nucleoplasm from the lumen of the nuclear envelope, whereas the outer leaflet (ONM) contacts the cytoplasm. The space between the leaflets is ultimately continuous with the lumen of the endoplasmic reticulum. Both leaflets are continuous with the nuclear pore membrane that serves as an anchoring point for nuclear pore complexes (NPCs), which serve as conduits to mediate protein and RNA transport between the nucleus and cytoplasm.

The nuclear lamina lines the inner surface of the INM and is maintained in this orientation by interaction with both chromatin in the nucleoplasm, and integral membrane proteins specifically concentrated in the INM. How proteins are targeted to the INM has been the focus of active research for several years (Ellenberg *et al.*, 1997; Ostlund *et al.*, 1999; Soullam and Worman, 1993). The leading "diffusion and retention" model proposes that proteins destined for the nuclear membrane are translated by membrane-bound ribosomes, and become integrated into the ER membrane. Proteins then migrate laterally towards the nuclear pore membrane. Those with extraluminal domains greater than 60–70 kD are excluded from the inner nuclear membrane, presumably because the bulky domain interferes with migration past the nuclear pore membrane (Soullam and Worman, 1995). Upon passing the nuclear pore membrane, the nucleoplasmic (or extraluminal) domain is in a position to interact with components of the nuclear lamina. For proteins such as lamin B receptor, emerin, and lamin associated proteins 1 and 2, interaction with lamina components essentially anchors the proteins at the INM, completing the targeting mechanism. For most proteins, however, a nucleoplasmic ligand to retain them at the inner nuclear membrane does not exist, so the proteins eventually migrate anterograde past the nuclear pore membrane into the ONM. Such proteins presumably continue in an anterograde direction to the ER, Golgi and beyond. Thus, most integral membrane proteins do not accumulate to high levels within the inner nuclear membrane, although they would be expected to localize there at least transiently.

The nuclear lamina is required for maintaining the structure of the nucleus and has been shown to line the entire inner surface of the nuclear membrane (Belmont *et al.*, 1993). The lamina is also required for functions normally associated with chromatin such as transcription and DNA replication (Gruenbaum *et al.*, 2000). The precise structure of the nuclear lamina is not well understood. Although many components of the lamina have been identified, understanding how these proteins interact to form higher order structures is complicated by the molecular complexity and redundancy of many of the interactions. What is clear is that most of the integral proteins within the INM that have been studied (such as lamin B receptor

and emerin) have been shown to bind lamins. Lamins are type V intermediate filaments and the nuclear lamina contains polymers of lamins A, B and C (McKeon *et al.*, 1986; Stuurman *et al.*, 1998). Lamins A and C are derived from splice variants of *LmnA* transcripts, whereas lamin B1 and lamin B2 are derived from different genes (Fisher *et al.*, 1986; Hoger *et al.*, 1988, 1990; Lin and Worman, 1993). As a result of RNA splicing, the C-terminus of Lamin C contains 6 unique amino acids that replace an isoprenylation motif and the C-terminal 90 amino acids of lamin A.

Lamins, like most intermediate filaments, contain globular head and tail domains separated by a coiled-coil helical rod-like domain that contributes the rigidity and most of the length of the filament. Lamins dimerize as a result of the intertwining of the rod domains, and larger filaments are produced upon multimerization of lamin dimers through the interactions of the rod domains of different protomers. The tail domain can form an immunoglobulin-like fold that likely interacts with chromatin (Dhe-Paganon *et al.*, 2002; Stierle *et al.*, 2003; Taniura *et al.*, 1995).

Envelopment at the nuclear membrane

Herpesvirus nucleocapsids are unique in virology because their nucleocapsids bud through the inner nuclear membrane (INM) to obtain a virion envelope. As a result of the envelopment reaction, the nascent virions accumulate between the inner and outer leaflets of the nuclear membrane. The nuclear membrane in sites that accommodate budding are more electron dense than other regions of the nuclear membrane, suggesting they bear a high density of proteins. Such envelopment sites are readily observed by electron microscopy along the inner nuclear membranes of HSV infected cells. While only a small subset of HSV proteins has been examined in this regard, at least proteins encoded by U_L 11 (a peripheral membrane protein), U_L 31, U_L 34, and glycoproteins B, D and M become incorporated into perinuclear virions and are present at the INM (Baines *et al.*, 1995; Jensen and Norrild, 1998; Reynolds *et al.*, 2002; Torrisi *et al.*, 1992; J. D. Baines, Jacob and B. Roizman, unpublished data). Because no HSV integral membrane protein is predicted to contain an extraluminal domain that exceeds the size that can preclude migration past the nuclear pore membrane (McGeoch *et al.*, 1988), it is likely that additional HSV integral membrane proteins will be discovered along the inner nuclear membrane. It is also likely that at least some tegument proteins are present at the INM, and that some associate with the nucleocapsid before they engage envelopment sites at the INM. Which of the many tegument proteins become targeted to virions in this manner has not been defined.

A critical question is whether the virions that accumulate in the perinuclear space are infectious. If so, it would seem advantageous to the virus because virions are often abundant in the perinuclear space late in infection, and would therefore represent a source of infectious virus upon immune-mediated cytolysis. The critical experiment to determine whether these virions are infectious has not been performed. Two seemingly disparate pieces of evidence argue both for and against: (i) cells infected with a virus encoding a truncated U_L20 gene fused to $U_L20.5$ contain abundant particles that appear to accumulate in the perinuclear space and these virions are infectious (Baines *et al.*, 1991); (ii) cells infected with a virus lacking U_S3 accumulate virions aberrantly in the perinuclear space, and there is a delay in the onset of infectious virus production (Reynolds *et al.*, 2002). Thus, it would seem that, unless an unforeseen defect in INM envelopment is also mediated by U_S3, these virions are not infectious until they exit the perinuclear space.

Studies of viral mutants lacking the U_L11, U_L31, U_L34, U_L37, and U_L53 (gK) proteins have shown that these proteins either facilitate or are required for nucleocapsid envelopment at the INM (Baines and Roizman, 1992; Desai *et al.*, 2001; Jayachandra *et al.*, 1997; Roller *et al.*, 2000). Except for U_L11 and U_L37 which are always dispensable, these proteins are essential for envelopment in different circumstances. Glycoprotein K is especially important for envelopment in quiescent cells (Jayachandra *et al.*, 1997). While U_L31 and U_L34 are essential for envelopment in Vero and Hep2 cells, U_L31 is dispensable for viral replication in rabbit skin cells (Chang *et al.*, 1997; Liang *et al.*, 2004; Roller *et al.*, 2000). Thus it seems likely that functions of the host cell can sometimes complement the functions of gK, and U_L31.

The U_L31/U_L34 protein complex and the nuclear lamina

The U_L31 protein of HSV-1 is a phosphoprotein that contains a bipartite nuclear localization signal at codons 8–25 and is predominantly intranuclear when transiently expressed in the absence of other viral proteins; in infected cells, however, the protein associates with the nuclear rim (McGeoch *et al.*, 1988; Reynolds *et al.*, 2001; Zhu *et al.*, 1999). Immunogold electron microscopy has revealed that the U_L31 protein associates with both leaflets of the nuclear membrane and that this association is dependent on U_L34 protein (Chang and Roizman, 1993; Reynolds *et al.*, 2001; Reynolds *et al.*, 2002). It was noted that U_L31 protein, like other components of the nuclear matrix, is resistant to extraction with detergent, DNAse and high salt (Chang and Roizman, 1993). Association with the nuclear matrix,

coupled with the distribution at the nuclear rim, strongly suggest that U_L31 protein integrates into the nuclear lamina of infected cells, components of which display similar resistance to extraction (Chang and Roizman, 1993; Reynolds *et al.*, 2001).

In vitro, U_L31 protein binds GST fusion proteins containing U_L34, and lamin A amino acids 369 to 633 (Reynolds *et al.*, 2004). The solubility of U_L31 protein under various extraction conditions strongly parallels the solubilization of lamin A/C, further supporting an in vivo interaction between these proteins. The presence of U_L31 protein in HSV-infected Vero cells reduces immunoreactivity of an epitope located between amino acids 369–519 of lamin A/C, suggesting that U_L31 directly or indirectly induces a conformational change in lamin A/C that masks the epitope. This region of lamin A/C includes the globular tail domain that has been shown to form an immunoglobulin-like fold that may interact with chromatin (Dhe-Paganon *et al.*, 2002; Stierle *et al.*, 2003). Thus, it is possible that U_L31 may compete for sites on lamin A/C that normally interact with chromatin. Such an interaction could conceivably play a a role in the U_L31-dependent HSV-mediated displacement of chromatin from regions of the INM (Simpson-Holley *et al.*, 2004).

Remarkably, overexpression of the U_L31 protein is sufficient to completely displace lamin A/C from the nuclear rim and cause it to colocalize with U_L31 protein within nucleoplasmic aggregates (Reynolds *et al.*, 2003). Similar lamina – disrupting activity has been seen upon overexpression of lamin A head or tail domains (Izumi *et al.*, 2000). The mechanism by which overexpressed U_L31 mediates lamin displacement is unclear. It is possible that this displacement reflects a competition between domains of U_L31 protein and lamin A/C that mediate: (i) anchoring of the lamina to the nuclear membrane or (ii) polymerization of lamin A/C filaments. However, such dramatic lamin A/C displacement is not observed in infected cells. Reasons include the possibilities that: (i) the distribution of U_L31 protein is restricted to the nuclear rim by its association with the U_L34 protein within the nuclear membrane, thus limiting its diffusion within the nucleus (Reynolds *et al.*, 2001), and (ii) the levels of U_L31 protein in infected cells are lower when expression is driven by the U_L31 promoter as opposed to the CMV early promoter/enhancer used to drive transient expression. If the ability of U_L31 to displace lamins reflects its utility as a lamin depolymerizing agent during infection, it seems more likely that in infected cells the U_L31 protein would serve to act within discrete regions of the nuclear membrane rather than globally. Limiting the destructive capacity of U_L31 protein to a local region may thus avoid more global nuclear disruption and preserve nuclear functions for optimal virus production.

U_L34 protein is likely a type II integral membrane protein, with the bulk of its 275 residue protein protruding into the nucleoplasm. Only the last five amino acids are predicted to reside between the lamella of the nuclear membrane or lumen of the endoplasmic reticulum (i.e., the perinuclear space). The U_L34 protein is necessary for nucleocapsid envelopment at the inner nuclear membrane in most cells tested including Vero and Hep2 cells. The U_L34 protein directly interacts with the U_L31 protein, and is essential for proper targeting of the latter to the nuclear membrane (Reynolds *et al.*, 2001). Changes in various charged clusters of U_L34 do not disrupt the interaction with U_L31 protein suggesting a hydrophobic region of the U_L34 protein may be responsible for the interaction or multiple areas interact (Bjerke *et al.*, 2003). Further work has indicated that amino acids 137–181 of UL34 protein are necessary and sufficient to interact with UL31 protein in the context of the infected cell (Liang and Baines, 2005). In the absence of U_L34, the U_L31 protein is more susceptible to degradation by the proteosome, and U_L31-specific epitopes localize mostly in the nucleoplasm (Ye and Roizman, 2000). In the reverse situation, the U_L31 protein is necessary for exclusive localization of U_L34 protein to the nuclear rim (Reynolds *et al.*, 2001).

Taken together, it is likely that the U_L31 and U_L34 proteins form a complex that is targeted to the nuclear rim. In many cell types, this targeting requires expression of both proteins. In rabbit skin cells, however, U_L34 protein localizes to the NM of infected cells even in the absence of U_L31 (Liang *et al.*, 2004). Thus, in certain situations, host cell functions are sufficient to properly target U_L34 protein to the NM.

The similarity of the U_L31/U_L34 protein complex to coupled pairs of lamin receptors and lamins is striking. For example, emerin, like U_L34 protein, is a type II integral membrane protein that interacts with lamin A/C (Clements *et al.*, 2000). Like lamin A/C, U_L31 integrates into the nuclear lamina and becomes associated with the nuclear membrane via binding its cognate lamin receptor (in this case U_L34 protein) at the nuclear membrane. Thus the U_L31/U_L34 protein protein complex is retained efficiently and invariantly at the nucleoplasmic surface of the INM.

Budding from the nuclear membrane

A prominent hypothesis is that the U_L31 and U_L34 proteins are retained at the inner nuclear membrane to engage nucleocapsids during envelopment. Support for

this hypothesis includes the observations that (i) U_L34 protein can interact with ICP5, the major capsid protein (Ye *et al.*, 2000), and (ii) both the U_L31 and U_L34 proteins are incorporated into virions that accumulate between the lamellae of the nuclear membrane. The latter indicates that the U_L31 and U_L34 proteins, directly or indirectly, interact with nucleocapsids in vivo (Reynolds *et al.*, 2002). Neither the U_L31 or U_L34 proteins are detectable in extracellular virions by electron microscopic immunogold staining. Thus, previous observations that the U_L34 protein was present in immunoblots of virions prepared from cytoplasmic lysates probably reflects the presence of at least some perinuclear virions in the cytoplasmic virion preparations (Purves *et al.*, 1992). It is presumed that the U_L31 and U_L34 proteins are released from the nascent virion envelope when this fuses with the outer nuclear membrane and the de-enveloped nucleocapsid is released into the cytoplasm. More detailed information on the cytoplasmic egress pathways of alphaherpesviruses is indicated in Chapter 12 of this volume.

US3: a kinase that phosphorylates U_L34 and U_L31 proteins

The U_L34 and U_L31 proteins are phosphorylated by the protein encoded by U_S3 (Purves *et al.*, 1991; Kato *et al.*, 2005; Poon and Roizman 2005). The U_S3 protein is not essential for growth of HSV in cell culture, but in the absence of U_S3, the onset of production of infectious virus is slightly delayed, and peak infectious titers are reduced about 30-fold, depending on the cell line (Purves *et al.*, 1987; Reynolds *et al.*, 2002). In the absence of U_S3, the architecture of the nuclear membrane is altered such that the membrane contains enveloped virions within round punctate extensions of the nuclear membrane. The virions in these regions also contain U_L31 and U_L34 proteins. It has been speculated that the presence of virions in these punctate nuclear membrane extensions (for simplicity of discussion, here termed NM evaginations) reflects a delay in egress from the perinuclear space (Klupp *et al.*, 2001; Reynolds *et al.*, 2002). The formation of NM evaginations is precluded by U_S3-encoded protein kinase activity (Ryckman and Roller, 2004). Interestingly, NM evaginations do not occur in cells infected with viruses bearing mutations that obviate U_L34 phosphorylation by U_S3. Thus, substrate(s) of the U_S3 kinase other than U_L34 protein are relevant to these particular NM perturbations. The identity of these novel substrate(s) is of considerable interest, and U_L31 is a lead candidate to explain the effects of U_S3 on virion envelopment at the INM .

gK

The localization of gK at the ultrastructural level has yet to be determined, largely because few epitopes in this mostly hydrophobic protein are recognized by available antibodies. In monolayers of tightly packed Vero cells infected with a U_L53 deletion mutant, nucleocapsids accumulate in the nucleus and few are detected in the cytoplasm, suggesting that at least under some conditions, gK plays an important role in nucleocapsid envelopment at the INM (Jayachandra *et al.*, 1997). How the protein facilitates nucleocapsid envelopment is not known.

U_L11

The U_L11 protein, composed of 96-residues, is both myristoylated and palmitoylated (Loomis *et al.*, 2001; MacLean *et al.*, 1989). Both modifications contribute to the association with membranes whereas an acidic cluster mediates trafficking from the plasma membrane to the Golgi apparatus in uninfected cells (Loomis *et al.*, 2001). Cells infected with the U_L11 deletion virus produce infectious virus at levels 100–1000-fold below those of wild-type viruses (Baines and Roizman, 1992). The most striking abnormalities in cells infected with this mutant are abundant unenveloped nucleocapsids in the cytoplasm and abutting the INM. These observations suggest roles in nucleocapsid envelopment at the nuclear membrane or completion of the envelopment reaction, and a second defect in cytoplasmic egress. The latter could reflect a defect that causes de-envelopment or one that precludes re-envelopment at cytoplasmic membranes. Although the U_L11 protein is targeted to the INM and cytoplasmic membranes of infected cells, the protein localizes primarily to the Golgi apparatus in uninfected cells (Baines *et al.*, 1995; Loomis *et al.*, 2001). The disparity between localization in infected and uninfected cells suggests that other infected cell proteins, or alteration of membranes or membrane trafficking by HSV helps mediate trafficking of the U_L11 protein to the nuclear membrane.

U_L37

Unlike many tegument proteins, the low U_L37 protein copy number in the virion tegument is invariant (McLaughlin, 1997), suggesting that it binds a repetitive structure on the nucleocapsid, and might serve as a limiting factor for tegument formation. A likely repetitive feature of nucleocapsids, and possible binding point of UL37 protein, would include capsid pentons which are structurally distinct from hexons and are believed to serve as anchor points for the

tegument (Zhou *et al.*, 1999). In pseudorabies virus, the ortholog of U_L37 has been termed a primary tegument protein to indicate its presence in the tegument layer most intimately assocated with the surface of the nucleocapsid (Mettenleiter, 2002). Deletion of U_L37 does not greatly affect production of nucleocapsides, but causes most of these to remain in the nucleus at time after infection when large amounts of cytoplasmic particles would be expected (Desai *et al.*, 2001). Thus U_L37 is ultimately dispensable for nucleocapsid envelopment, but greatly facilitates the process. Taken together, the data suggest the (perhaps oversimplified) hypothesis that U_L37 protein association with intranuclear capsids serves to bridge the nucleocapsid to a U_L37-interacting protein in the envelopment apparatus at the INM, but how pU_L37 becomes incorporated into the tegument, or whether it interacts with appropriate envelopment proteins to accomplish a bridging function have yet to be determined.

Model of nucleocapsid envelopment at the INM

A model for nucleocapsid envelopment at the nuclear membrane that is consistent with the above data is as follows. (i) The U_L31 protein is imported through the nuclear pores via its N-terminal nuclear localization signal. The protein interacts with the globular tail domain of lamin A/C and thereby becomes incorporated into the nuclear lamina. (ii) The U_L34 protein becomes incorporated into the ER membrane via its C-terminal transmembrane domain with the bulk of the protein located in the cytoplasm. The protein migrates past the nuclear pore complex and into the INM where it engages U_L31 protein located in the lamina, causing it to become anchored in the INM. (iii) Chromatin and associated lamins are displaced from regions containing U_L31 and U_L34 proteins and the conformation of lamins is significantly altered directly or indirectly by the U_L31/U_L34 protein complex, resulting in localized thinning of the lamina. (iv) several other viral proteins including gD, gB, gM, gK, and U_L11 protein are recruited to patches on the nuclear membrane that correspond to areas of lamin thinning. These regions also contain the U_L31 and U_L34 protein complex. (v) Nucleocapsids engage at least the U_L34 protein to initiate nucleocapsid budding at the INM. As a result of the budding reaction, the U_L31, U_L34, and U_L11 proteins, along with gB, gD, gK, and gM (as well as other unidentified proteins) become incorporated into nascent virions (vi) The envelopes of virions within the perinuclear space fuse with the outer nuclear membrane (ONM). The U_L31 and U_L34 proteins are left at the ONM, and the de-enveloped nucleocapsid proceeds into the cytoplasm to receive another membrane from a cytoplasmic organelle.

Although the above model is consistent with current observations, it is likely that certain aspects (and future surprises) will invite revision as more data accumulates. The usefulness of the model lies in its many testable predictions, and it is hoped that such a paradigm will invite vigorous experimentation in the future.

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12

The egress of alphaherpesviruses from the cell

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A commonly accepted concept in herpesvirology holds that herpesvirions are formed by budding of nucleocapsids at the inner nuclear membrane and the enveloped virions are released into the perinuclear space (see Chapter 13). This is a closed compartment that virions need to exit, in order to reach the extracellular space and start a new infection cycle. How alphaherpesviruses accomplish this goal is a controversial issue. Of the two pathways of virus exit proposed, the single envelopment and the double envelopment, also referred to as de-envelopment–re-envelopment, each has evidence and supporters in the literature (the topic has been covered in excellent reviews and papers (Enquist *et al.*, 1998; Skepper *et al.*, 2001; Johnson and Huber, 2002; Mettenleiter, 2002). Part of the uncertainties that still dominate this topic comes from the difficulties in interpreting static electron microscopy images. Thus cytoplasmic virions juxtaposed to curved vesicles were interpreted in some studies as budding virions, i.e., as evidence for secondary envelopment and for the deenvelopment-reenvelopment pathway. In other studies they were interpreted as virions undergoing fusion with encasing vesicles, i.e., as evidence of de-envelopment (Campadelli-Fiume *et al*, 1991: Roizman and Knipe, 2001). To solve these ambiguities, several approaches have been undertaken in recent years, including the generation of genetically modified mutants and cytochemistry.

In the single envelopment pathway, credited to a study by Johnson and Spear (Johnson and Spear, 1982) in which monensin was observed to block herpes simplex virus (HSV) glycoprotein maturation and to induce the accumulation of virions in large cytoplasmic vacuoles, virions leave the perinuclear space by becoming encased in vesicles–vacuoles formed by the outer nuclear membrane (Fig. 12.1, left pathway). At this stage they carry immature oligosaccharides in their glycoproteins and glycolipids. The virion-encasing vesicles then travel along the exocytic or secretory pathway, and interact with membranes of the exocytic pathway, mainly the Golgi apparatus, leading to a modification in their content of glycosyl transferases and glycosidases that results in the *in situ* maturation of the oligosaccharides of viral glycoproteins. The mature virions are then released in the extracellular space by fusion of the virion-encasing vesicle with the cytoplasmic face of the plasma membrane. In this pathway the virion maintains the tegument acquired in the nucleus as well as the envelope acquired at the inner nuclear membrane, hence the glycoprotein species present in the initial envelope do not change, but their oligosaccharidic moieties are subject to maturation.

In the de-envelopment–re-envelopment pathway, originally proposed by Stackpole in a study of frog herpesvirus (Stackpole, 1969), the envelope of the virions present in the perinuclear space fuses with the outer nuclear membrane (de-envelopment), thus releasing the nucleocapsids into the cytoplasm. The de-enveloped nucleocapsids acquire a tegument in the cytoplasm and undergo a secondary envelopment (re-envelopment) by nucleocapsid budding into a *trans*-Golgi compartment or *trans*-Golgi network (TGN), or, into an endosomal compartment (Harley *et al.*, 2001) (Fig. 12.1, right pathway). As the virus buds from these membranes, the membrane gives rise simultaneously to the envelope and to a vesicle that surrounds the enveloped virion. The final release of the virions into the extracellular space takes place by fusion of the virion-encasing vesicle with the cytoplasmic face of the plasma membranes, just as it occurs in the single envelopment pathway. In this pathway the virus acquires in the cytoplasm a tegument and a secondary envelope, both of which may differ in protein composition from the ones acquired at the primary envelopment.

The key differences between the two routes are (i) the number of envelopes that the virus acquires: one

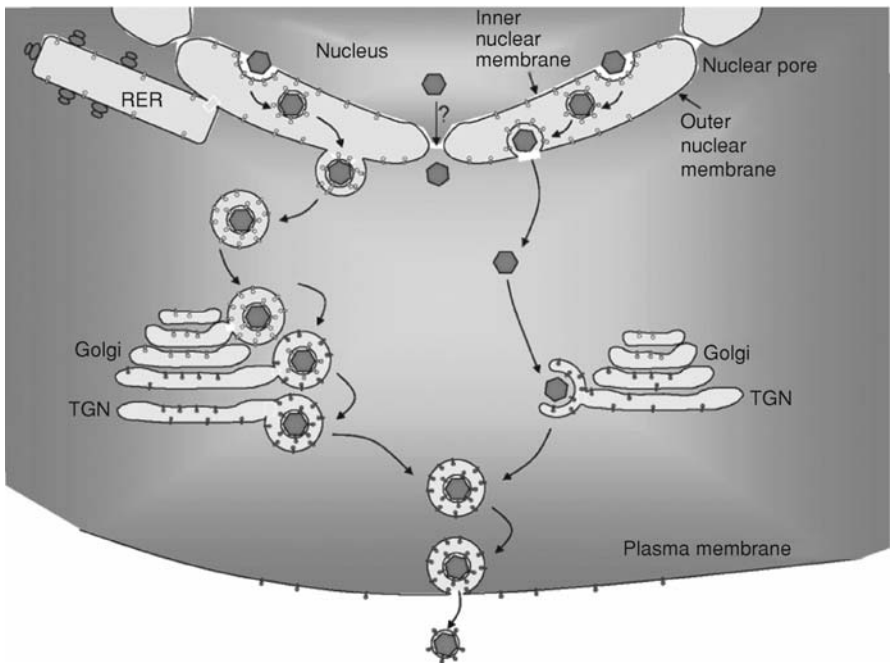

Fig. 12.1. Schematic drawing showing the two alternative pathways of alphaherpesvirus egress from infected cells. The single envelopment pathway is depicted to the left, and the double envelopment, or de-envelopment-re-envelopment is depicted to the right of the illustration. The schematic drawing does not shows the gross ultrastructural modifications of the Golgi apparatus and TGN. Perinuclear virions and nuclear membranes are decorated with glycoproteins of different color than virions at the level of the Golgi apparatus and TGN, as well as extracellular virions, to emphasize that the oligosaccharide moieties of the viral glycoproteins are of the immature type in early exocytic compartment, but are of the mature type in the late exocytic compartments and in extracellular virions. The drawing considers also the possibility that nucleocapsids exit the nucleoplasm through modified nuclear pores, without transiting through the perinuclear lumen. (Drawing by courtesy of L. Menotti.) (See color plate section.)

in the single envelopment, two in the de-envelopment-re-envelopment pathway; the composition of the two envelopes may well differ one from the other; (ii) The significance of the capsids in the cytoplasm. In the single envelopment pathway, the cytoplasmic nucleocapsids are dead-ends that result from fusion of the envelope with the membrane of the virion-encasing vesicles (Campadelli-Fiume *et al.*, 1991). In the de-envelopment–re-envelopment pathway, they are the key players for the secondary envelopment. (iii) The site of tegument assembly, which is necessarily the nucleus in the single envelopment pathway, and can be either the nucleus or the cytoplasm in the double envelopment egress. In the latter case, the tegument may even be absent from perinuclear virions.

In recent years there has been a growing consensus in favour of the de-envelopment–re-envelopment pathway for egress of both HSV and varicella zoster virus (VZV) (Jones and Grose, 1988; Enquist *et al.*, 1998; Wang *et al.*, 2001; Mettenleiter, 2002). As some crucial questions concerning this route remain unsolved, both models are presented here, along with the evidence in favour of or against each of the pathways. For an elegant and in-depth analysis of both pathways, and of strengths and weaknesses of the lines of evidence, also see Enquist *et al.* (1998).

HSV

Single envelopment pathway

Evidence and arguments in favor

A major virtue of this pathway is its simplicity. It became widely accepted some years ago, and was supported by three lines of evidence.

(i) When analyzing the types of oligosaccharides that are present in the glycoproteins and in the glycoplipds of the virion envelope, the model predicts that the perinuclear virions carry immature glycomoieties, the extracellular virions carry mature glycomoieties, and the cytoplasmic virions carry both intermediate and mature glycomoieties. This intermediate type of glycomoieties cannot

be present in virions formed in the de-envelopment–re-envelopment pathway, unless the secondary envelopment takes place at the *cis*- or *medial*-Golgi. If this is the case, then the question arises "how does the virion travel from *cis*- or *medial*-Golgi to farther compartments of the exocytic pathway in order to obtain its final envelope with mature oligosaccharides?" When the HSV oligosaccharides were characterized in a cytochemical study, it was found that perinuclear virions carry immature oligosaccharides, the intracytoplasmic virions carry both intermediate and mature types of oligosaccharides, and extracellular virions carry exclusively mature oligosaccharides (Di Lazzaro *et al.*, 1995).

(ii) A similar line of reasoning applies to the significance of the markers of the *cis*- and *medial*-Golgi in extracellular virions. It is a well-known phenomenon that viral envelopes carry cellular proteins that are constituents of the membranes where budding of virions occurred, or of the compartments which the virus transited. As a consequence, the cellular proteins that are present in the envelope of extracellular virions are indicators of the cellular membranes from which the envelope was derived, or through which the viruses transited. Specifically, if virions undergo a secondary envelopment at *trans*-Golgi or TGN, the extracellular virions are not expected to carry proteins typical of *cis*- or *medial*-Golgi. By contrast, if these markers are present in the extracellular virions, two mutually exclusive implications are possible, i.e., either the virions transited through *cis*- or *medial*-Golgi, as is the case for the single envelopment pathway, or budding took place at *cis*- or *medial*-Golgi. As discussed above, if this is the case, the same question as above arises: "How does the virion move from *cis*- *medial*-Golgi to *trans*-Golgi or TGN?" A detailed immunocytochemical study, summarized below, has been conducted in neurons, and showed that extracellular virions carry giantin and mannosidase II, two markers typical of *cis*- and *medial*-Golgi, respectively (Miranda-Saksena *et al.*, 2002).

(iii) A further issue centers on the glycoprotein composition of the perinuclear virions, and particularly whether the glycoproteins required for virion infectivity are acquired during envelopment at the inner nuclear membrane. It is a well established notion that HSV infectivity requires the four glycoproteins gB, gD, gH, gL (see Chapter 7). Perinuclear virions were isolated from cells infected with a UL20-deletion virus, which induces the accumulation of virions in the perinuclear space. They are infectious, implying that they carry the four glycoproteins gB, gD, gH, gL (Baines *et al.*, 1991; Avitabile *et al.*, 1994a). The actual presence of the essential glycoproteins at nuclear membranes and at the perinuclear virions was established in *in situ* experiments for the two glycoproteins that were analyzed: gD and gB (Torrisi *et al.*, 1992; Skepper *et al.*, 2001; Miranda-Saksena *et al.*, 2002). All in all, the essential glycoproteins that were searched for have been detected in perinuclear virions, suggesting that, if the de-envelopment–re-envelopment takes places, it is not a requirement in order for the virus to obtain its asset of essential glycoproteins.

Evidence and arguments against

There are two major lines of evidence against the single envelopment model. They are: the presence of the UL31 and UL34 proteins at the nuclear membranes and at the perinuclear virions, and their concomitant absence from extracellular virions, and the lipid composition of the virion envelope (van Genderen *et al.*, 1994; Reynolds *et al.*, 2001, 2002). Both rest on the notion that the chemical composition of the virion envelope reflects the composition of the membrane where nucleocapsid budding took place.

(i) The UL31 protein is a matrix-associated phosphoprotein that localizes to the nuclear membranes in the infected cells, and when coexpressed with the UL34 protein (see Chapter 13). The UL34 protein is a predicted type-2 membrane-bound phosphoprotein with the bulk of the protein constituting the endodomain exposed to the interior of the nucleus. It requires the UL31 protein for localization at the nuclear envelope. The two proteins are required for virus envelopment. Specifically, electron microscopic analyses show that morphogenesis of a UL34 gene deletion virus proceeds to the point of formation of DNA-containing nuclear capsids, but enveloped virus particles in the cytoplasm or at the surface of infected cells are absent, suggesting that the UL34 protein is essential for efficient envelopment of capsids. The phenotype of the UL31 gene deletion virus is similar (Roller *et al.*, 2000; Ye and Roizman, 2000). Remarkably, both proteins are present at perinuclear virions but absent from the extracellular virions, providing evidence that the primary envelope differs in composition from the envelope of extracellular virions. This strongly argues in favour of the de-envelopment-re-envelopment pathway (Reynolds *et al.*, 2001, 2002). The same localization is observed with pseudorabies virus (PrV) UL31 and UL34 proteins (Fuchs *et al.*, 2002). An alternative explanation for the absence of UL31 and UL34 proteins from the extracellular virions would be that a specific protease degrades these two proteins during virus maturation, in analogy to what happens for the scaffolding protein VP26, or, less likely, that epitopes become masked.

(ii) The phospholipid composition was determined in infected cells fractionated into three fractions: a nuclear fraction (which contains nuclei and virions at perinuclear space), a cytoplasmic fraction (which contains the

cytoplasmic membranes, the intracytoplasmic virions and the plasma membranes), and a fraction consisting of extracellular virions. It was found that the phospholipid composition of extracellular Herpes Simplex virions and of the cytoplasmic fraction differs from that of nuclei, in that the former contains threefold higher concentrations of sphingomyelin and phosphatidylserine, lipids that are typically enriched in the Golgi apparatus and plasma membrane (van Genderen *et al.*, 1994). This difference implies that the lipid composition of the envelope acquired at the inner nuclear membrane differs from that of extracellular virions. It has been interpreted as evidence in favour of the de-envelopment–re-envelopment pathway (Enquist *et al.*, 1998), even though alternative mechanisms for the modification in lipid composition may be envisioned, e.g., exchange of lipids between the virion envelope and the Golgi membranes during exocytosis in the single envelopment pathway (van Genderen *et al.*, 1994).

De-envelopment–re-envelopment pathway

Evidence and arguments in favor

A major virtue of the double envelopment route of egress is that the separate transport of nucleocapsids and glycoproteins, and their assembly into virions at the cell periphery seems a rational way of transport in the neuron: a key host cell for HSV. In addition to the localization of the UL31 and UL34 proteins, and to the lipid composition of the virion envelope, discussed above, three lines of evidence argue in favour of a double envelopment process, namely the separate transport of nucleocapsids and glycoproteins in neuronal axons, the phenotype of mutants carrying deletions in tegument genes or multiple deletions in glycoprotein genes with accumulation of unenveloped nucleocapsids in the cytoplasm, and the differential distribution of a form of gD retargeted to the endoplasmic reticulum.

(i) A first line of evidence for separate transport of capsids and viral glycoproteins was provided in an electron microscope study of HSV assembly in neurons. Capsids were observed migrating in anterograde direction within axons, whereas the viral glycoproteins (gD) were observed in vesicles which do not colocalize with nucleocapsids, suggesting that virion assembly in axons represents a final step that occurs at the axon end (Penfold *et al.*, 1994).

(ii) Tegument assembly: the tegument is one of the most complex and least understood components of the virion, in terms of structure, assembly and role in virus entry and in virion morphogenesis. Functionally, it is analogous to the matrix layer of other viruses, as it connects the capsid to the intravirion tails of the envelope glycoproteins. Its role is twofold. First, it delivers into the cytosol of the infected cell virion components which are immediately available to the viral metabolism, before the onset of viral protein synthesis. These components facilitate the initiation of infection. Two such examples are *alpha*-TIF (α-*trans*-inducing factor), also named VP16, and *vhs* (virion host shut off), the product of UL41 gene (Batterson *et al.*, 1983; Read and Frenkel, 1983; Campbell *et al.*, 1984).The second function is structural (Mossman *et al.*, 2000). In terms of composition, it is made of almost 20 proteins (see Chapter 7). Despite its amorphous appearance, the tegument appears to contain an inner and an outer layer, of different composition. For both HSV and PrV, the inner layer contains the products of the UL36 (VP1/2) and UL37 genes. The UL36 protein interacts with the major capsid protein, ICP5, which forms both pentons and exons. The outer layer includes major components, UL48-αTIF-VP16, UL49-VP22, *vhs*, and minor components, UL11, UL13-PK (protein kinase), UL14, UL21, UL46-Vp11/12, UL47-VP13/14, UL51, UL56, US3-PK, US10, US11.

Studies of tegument assembly were interpreted as evidence that the site of assembly is the cytoplasm. They fall into two series: electron microscopic analysis and the phenotype of mutant viruses deleted in the tegument-encoding genes.

Work performed mainly with PrV indicates that intranuclear nucleocapsids do not contain a well-defined tegument, detected as an electrondense layer surrounding the capsid, whereas extracellular virions and cytoplasmic nucleocapsids contain an electrondense tegument (Mettenleiter, 2002). In line with these observations, in live virus-infected cells the tegument protein VP22 is observed almost exclusively in the cytoplasm, favoring the cytoplasm as the site of tegument assembly (Elliott and O'Hare, 1999).

In terms of deletion mutant viruses, the UL36 protein appears to play a critical role in tegument assembly, since in its absence capsids acquire the envelope at the inner nuclear membrane, are subsequently translocated into the cytoplasm, but do not mature into enveloped virus and do not exit the cell. Also the deletion of HSV UL37 gene abrogates virus maturation and induces a phenotype similar to that induced by the UL36 gene deletion (Desai, P. J., 2000; Desai, P. *et al.*, 2001). The physical interaction between UL36 and UL37 proteins has been demonstrated in PrV (Klupp *et al.*, 2002). These phenotypes have been interpreted to mean that a defect in tegument assembly hampers the secondary envelopment with consequent accumulation of cytoplasmic nucleocapsids, although they do not formally prove it (Desai, P. J., 2000).

In contrast to the effect of UL36 and UL37 deletions, virion morphogenesis is not hampered in a VP22 deletion mutant HSV, nor in a number of deletion mutant viruses

in the genes encoding other tegument proteins (Pomeranz and Blaho, 2000; Mettenleiter, 2002). A defective phenotype is however observed in the deletion mutant virus of α-TIF, a phenotype complicated by the fact that the protein has two functions, i.e., it is a potent transactivator of α-genes and is required for the structural integrity of the tegument (Batterson *et al.*, 1983; Campbell *et al.*, 1984; Ace *et al.*, 1988; Mossman *et al.*, 2000). The observation that most of the deletion mutants in tegument genes fail to induce a defect in virus assembly and production has been interpreted as evidence that tegument proteins play redundant functions, and that the absence of a single tegument protein does not hamper tegument assembly (Mettenleiter, 2002).

An immunocytochemical study of tegument assembly and envelope acquisition in rat dorsal neurons showed that the site of HSV tegument assembly is the cytoplasm of the neuronal cell body and that major sites of envelope acquisition are the vesicles of the Golgi and TGN. Evidence rested on the finding that the tegument proteins VP13/14, VP16, VP22, and US9 were readily detected in the nucleus, but almost absent from the budding virions at the nuclear membranes and from virions in the perinuclear space. By contrast, they were abundant on cytoplasmic unenveloped and enveloped nucleocapsids and in extracellular virions (Miranda-Saksena *et al.*, 2002). Of note, the same pattern of labelling was observed for gD. Altogether, the results of this study were interpreted as evidence that the pathway of virion egress from the cell body of neurons does not differ from the pathway of egress from axons during anterograde transport, and follows the de-envelopment–re-envelopment pathway. As mentioned above, in this same study the extracellular virions and the cytoplasmic vesicles also labelled for markers of *cis*- and *medial*-Golgi and of TGN. The significance of this finding was not discussed (Miranda-Saksena *et al.*, 2002).

(iii) Multiple deletion of glycoprotein genes, especially non essential glycoproteins. Practically all HSV glycoproteins, both essential and non-essential, have been deleted singly or in groups, and none of the deletion viruses is defective in virus transport out of the perinuclear space (Longnecker *et al.*, 1987; Longnecker and Roizman, 1987; Cai *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.*, 1992; Baines and Roizman, 1993; Roop *et al.*, 1993; Dingwell *et al.*, 1994), suggesting that no single glycoprotein plays a critical role in virus exocytosis. The exceptions are the gK- and UL20-deletion mutant viruses discussed below. In contrast, viruses carrying multiple deletions in glycoprotein genes exhibited defects in virus morphogenesis, cumulatively suggesting that gE, gI and gD participate in secondary envelopment and that they act in a redundant manner, such that viruses carrying single or double deletions do not exhibit a marked phenotype, whereas viruses carrying a triple deletion do (Farnsworth *et al.*, 2003). Specifically, gE and gI play a role in the cell-to-cell spread of HSV, visible in untransformed cells (see below). gM is an abundant glycoprotein conserved in all human herpesviruses. While its conservation argues for a role of the glycoprotein, a deletion mutant virus has no phenotype in cell culture. In PrV the simultaneous deletion of gE–gI and gM drastically inhibits plaque formation and replication, and induces the accumulation of nucleocapsids in cytoplasmic areas where tegument proteins accumulate (Brack *et al.*, 1999). This phenotype indicates that the deleted glycoproteins are cumulatively responsible for secondary envelopment. Following these observations, HSV deletion viruses in gE–gI or gE–gI–gM have been constructed. Remarkably, they exhibit no defect in growth, plaque formation, particle to PFU ratio, hence they carry none of the defects of the triple deletion PrV mutant (Browne *et al.*, 2004). In an independent work, HSV mutants carrying a double or a triple deletion in gD–gE or gD–gE–gI, respectively, exhibited severe defects in envelopment, detected as accumulation of a large number of unenveloped nucleocapsids in the cytoplasm (Farnsworth *et al.*, 2003). These aggregated capsids were immersed in an electron-dense layer that appeared to be tegument. Because none of the glycoproteins, when deleted singly, produced this phenotype, it was proposed that gD and the gE–gI act in a redundant fashion to enable the interaction of the virion envelope with tegument-coated capsids. In the absence of either one of these HSV glycoproteins, envelopment proceeds; however, without both gD and gE, or gE/gI, an inhibition of cytoplasmic envelopment is observed (Farnsworth *et al.*, 2003).

(iv) A form of gD carrying an endoplasmic reticulum (ER) retrieval motif. The rational of this study was to engineer into HSV a form of gD carrying an ER-retrieval motif. The ER-retrieved gD was expected to be present at the ER and the nuclear membranes, which are continuous with the ER, but not to travel along the exocytic pathway. Virions at the perinuclear space were expected to be decorated with gD; virions undergoing de-envelopment–re-envelopment were expected to exchange this envelope, so that extracellular virions would be devoid of gD (Whiteley *et al.*, 1999; Skepper *et al.*, 2001). When analyzed by immunocytochemistry, the perinuclear virions and the nuclear membranes were indeed decorated with the ER-retargeted gD, whereas the extracellular virions and the plasma membrane were not (Whiteley *et al.*, 1999; Skepper *et al.*, 2001). These results are consistent with a double envelopment route of egress. However, in the same study quantification

of the distribution of gD in wild-type virus-infected cells showed that the plasma membranes contained 20-fold less, or even lower amount of gD than the nuclear and cytoplasmic membranes (Skepper *et al.*, 2001). This contrasts with the detection of gD in approximately the same amounts at the nuclear membranes and at the plasma membranes in a fracture-label study, with the abundant detection of gD at the plasma membrane by immunofluorescence, and with the detection of gD more abundantly at the plasma membrane than at the nuclear membranes in neurons and raises the possibility that the degree of detection may be affected by the specific reactivity of the antibodies employed (Torrisi *et al.*, 1992; Miranda-Saksena *et al.*, 2002).

Evidence and arguments against

(i) The major weakness of the de-envelopment–re-envelopment pathway is the inability to explain how virions leave the perinuclear space (Campadelli-Fiume and Roizman, 2006). The pathway envisions that perinuclear virions fuse with the luminal face of the outer nuclear membrane, thus releasing the de-enveloped nucleocapsids in the cytoplasm. The glycoproteins necessary for the HSV fusion that leads to virus entry are the quartet of gB, gD, gH, gL (Cai *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.*, 1992; Roop *et al.*, 1993). These same glycoproteins are necessary and sufficient to induce cell–cell fusion when transiently expressed in transfected cells (Turner *et al.*, 1998) (see Chapter 7). Absence of each member of the quartet abolishes virion infectivity and fusion in the cell–cell fusion assay, but does not cause any defect in the release of (non-infectious) virions to the extracellular space. Paradoxically therefore, the perinuclear virions of the deleted viruses are able to carry out fusion with the outer nuclear membrane in the absence of each one of the known viral fusion glycoproteins.

To solve this conundrum, three alternative possibilities can be envisioned. First, some of the non-essential glycoproteins or membrane proteins which form the envelope of the perinuclear virions substitute for the fusion activity of the quartet. As a corollary, deletion mutants in these putative fusion proteins are expected to accumulate in the perinuclear space and be defective in the release of extracellular virus. Apart from the fact that a fusion activity has not been observed with ensembles of HSV glycoproteins other than the quartet (Turner *et al.*, 1998), deletion viruses have been produced for almost all of the non essential glycoproteins (gC, gE, gI, gJ, gG, gM), and they are not defective in the release of virions to the extracellular space (Longnecker *et al.*, 1987; Longnecker and Roizman, 1987; Cai *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.*, 1992; Baines and Roizman, 1993; Roop *et al.*, 1993; Dingwell *et al.*, 1994). This makes it unlikely that the non-essential envelope proteins carry out fusion with the outer nuclear membrane. The phenotype of two deletion viruses is interesting under this respect. They are a gK-minus and a UL20-minus virus (Baines *et al.*, 1991; Avitabile *et al.*, 1994b; Hutchinson and Johnson, 1995). The first-generation deletion viruses exhibit an accumulation of virions at the perinuclear space. The second-generation deletion mutants are still defective in virus egress, but the unenveloped nucleocapsids appear to accumulate in the cytoplasm rather than in the perinuclear space (Foster and Kousoulas, 1999; Foster *et al.*, 2004). This phenotype is consistent with the hypothesis that gK and/or UL20 proteins, singly or in association, enable fusion. Because these proteins accumulate at the ER, or in the Golgi, but only in minimal quantity, or not at all, in the plasma membrane, when expressed by transgenes (Avitabile *et al.*, 2003, 2004; Foster *et al.*, 2003), the possibility that they induce fusion at the nuclear membranes can not be tested.

Secondly, it can be envisioned that fusion of perinuclear virions with the outer nuclear membranes is carried out by cellular proteins, e.g. members of the v-SNARE and t-SNARE family. This possibility appears untenable because of the topology of these proteins, whose functional domains are located in the cytoplasmic face of the cytoplasmic vesicles. They would need to flip–flop to the luminal face, while maintaining their activity, and travel all the way to the outer and then to the inner nuclear membrane, in order for perinuclear virions to carry them in their envelope. Further yet, these proteins need a number of membrane-bound and soluble factors, all of which are absent from the perinuclear space.

All in all, the virus has to rely on viral fusion proteins other than those known to date in order to exit the perinuclear space, and an unconventional and so far totally elusive mechanism of fusion must be hypothesized in order to explain how virions leave the perinuclear space in the deenvelopment and re-envelopment pathway.

A third possibility is raised by the recent finding that in HSV-and BHV-infected cells the nuclear pores appear to be enlarged such that they allow the exit of nucleocapsids directly from the nucleoplasm to the cytoplasm (Wild and Engels, 2004; Leuzinger *et al.*, 2005).

(ii) A second weakness of the double envelopment pathway is the failure to explain how the de-enveloped nucleocapsids travel from the cytoplasmic face of the outer nuclear membrane to the Golgi or TGN. As discussed in Chapter 7, nucleocapsids that do not travel along microtubules move very slowly (see the calculations that have been present for incoming nucleocapsids), and therefore

the de-enveloped nucleocapsids need to travel along some kind of cellular routes for efficient transport. Transport along microtubules seems untenable since the microtubule architecture is dramatically modified in the course of infection, a modification that appears to be conserved in herpesviruses (Avitabile *et al.*, 1995). The infected cell microtubules form circular rings at the periphery of the cell, and seem rather unsuitable to export nucleocapsids from outside the outer nuclear membrane to the TGN.

Finally, as mentioned above, the presence of *cis*- and *medial*-Golgi markers in extracellular virions produced by neurons suggests that either these membranes were the site of secondary envelopment, or that virions have transited through these compartments, with exchange of components, as may happen in the single envelopment route of egress (Miranda-Saksena *et al.*, 2002).

Cell-to-cell spread

The prominent route by which HSV infection spreads in human tissues is cell-to-cell spread, i.e., the direct passage of progeny virus from an infected cell to an adjacent cell. This occurs at primary infection when progeny virus spreads from the primary infected cell to adjacent cells in the mucocutaneous tissue and then to axonal termini of sensory neurons (retrograde transport). It also occurs at reactivation from latency, when newly replicated virus spreads from the sensory neuron to the mucocutaneous tissue (anterograde transport). It is generally assumed that this mechanism of spread represents an immune evasion strategy, as it shields the virus from antibodies and cells of the immune system. The simplest models of cell-to-cell transmission in cell cultures are plaque formation and the infectious center assay. Some of requirements are relatively well characterized, and coincide with those for virus entry, i.e., the quartet of gD, gB, gH, gL and the presence of a gD receptor on target cells. In addition, gE and gI play a critical role.

gE–gI

gE and gI form a functional heterodimer (gE–gI), found both in infected cell membranes and in virion envelopes (Johnson *et al.*, 1988), an attribute conserved in VZV and PrV (Zuckermann *et al.*, 1988; Yao *et al.*, 1993). The role of the gE–gI complex was not recognized in early studies because single deletion mutants are not hampered in their replication, or in the rate at which extracellular virus particles enter cells, whether the virus is applied to the apical or basolateral surfaces of the cells (Dingwell *et al.*, 1994). The lack of phenotype of the single deletion mutants has been later ascribed to the transformed cells in which they were characterized; in cells that form extensive cell junctions, like normal human fibroblasts and epithelial cells, the mutants are compromised (Collins and Johnson, 2003). The deleted viruses are also severely attenuated in vivo, and fail to spread efficiently into and within specialized circuits of the nervous system (Dingwell and Johnson, 1998). Several mechanisms appear to regulate the gE–gI-mediated cell-to-cell spread. Thus, gE–gI facilitate the movement of HSV across the extensive junctions formed between epithelial cells, fibroblasts, and neurons in vivo, likely by sorting the newly assembled virions to lateral surfaces and cell junctions (Johnson *et al.*, 2001), and by promoting envelopment into vesicles that are sorted to epithelial cells of junctions (Johnson and Huber, 2002).

US9

Although PrV is beyond the scope of this chapter, the role of US9 protein is better illustrated in this system. US9 protein is critical in axonal transport and in interneuronal spread of PrV (Enquist *et al.*, 1998; Brideau *et al.*, 2000). Structurally, it is a phosphorylated type II membrane glycoprotein present in the lipid envelope of viral particles and in the infected cell *trans*-Golgi network in a unique tail-anchored topology. Its maintenance in the TGN region is a dynamic process involving retrieval of molecules from the cell surface, mediated by an acidic cluster containing putative phosphorylation sites and by a dileucine endocytosis signal (Brideau *et al.*, 1999). The role of US9 protein in transneuronal spread of PrV was inferred by the phenotype of US9-null mutants, which exhibited a defect in anterograde spread in the visual and cortical circuitry of the rat. Hence, the US9 protein functions together with gE, and gI to promote efficient anterograde transneuronal infection and in the directional spread in the rat central nervous system (Brideau *et al.*, 2000; Tomishima and Enquist, 2001). The phenotype of the US9-null virus is consequent to the ability of the protein to regulate the intracellular traffic of viral proteins in axons. Specifically, in US9-null mutant infections the viral membrane proteins fail to enter axons, while the capsids and tegument proteins do enter axons. These findings have been interpreted as evidence that virion subassemblies, but not complete virions, are transported in the axon, and consequently that the final assembly of virions takes place at the axon periphery (Tomishima and Enquist, 2001). Although a detailed characterization of HSV US9 is missing, the PrV and HSV proteins are likely to behave in a similar manner.

VZV

As compared to HSV, there have been relatively few studies dealing with the topic of VZV egress; they were performed mainly by transmission electron microscopy and immunocytochemistry and made use of a limited number of viral mutants. Indeed, the wealth of deletion and genetically modified viruses that characterizes the studies of HSV egress has no match in VZV. Cumulatively, these studies led to the conclusion that the pathway of VZV egress involves envelopment at the TGN, thus favouring the idea that the virus undergoes a de-envelopement–re-envelopment process.

Three glycoproteins, gE, gI, and gB, play a role in VZV egress. A major focus has been on gE, the most abundant envelope glycoprotein. By contrast with HSV gE, VZV gE is essential (Jones and Grose, 1988; Mo *et al.*, 2000, 2002; Moffat *et al.*, 2004). Electron microscopy showed that gE is absent from perinuclear virions, but present in TGN-derived membranes, and in the cytoplasmic and extracellular virions. The TGN membranes acquire a flattened "C" shape and are decorated with the tegument proteins on the concave face, which appears to serve as a budding site for envelopment (Gershon *et al.*, 1994). gE carries several structural motifs. A tyrosine-based motif in the C-tail acts as a determinant for TGN-targeting, and was interpreted as a driving element for secondary envelopment at TGN (Zhu *et al.*, 1996). A detailed mutational analysis of this domain indicates that proper subcellular localization and cycling of gE depend not only on the tyrosine-containing tetrapeptide related to endocytosis sorting signals, but also on a cluster of acidic amino acids containing casein kinase II phosphorylatable residues (Alconada *et al.*, 1996). gE is phosphorylated by the tegument ORF47 protein kinase; phosphorylation is critical for gE trafficking to the TGN and for its recycling from the plasma membrane (Kenyon *et al.*, 2002). As mentioned above, in the UL47(PK) deletion mutant the lack of gE phosphorylation results in a recycling back to the plasma membrane (Kenyon *et al.*, 2002).

As in HSV, gE forms a complex with gI, and the gE–gI complex is a determinant of VZV cell-to-cell spread, as well as of the maturation, endocytosis and recycling from the plasma membrane of both glycoproteins (Alconada *et al.*, 1998, 1999; Olson and Grose, 1998; Mo *et al.*, 2002); The role of gI in virion morphogenesis was investigated in cells infected with VZV mutants lacking gI, or a portion of gI ectodomain. It was observed that the TGN loses the ability to bind tegument proteins, a property correlated with an overall reduction in cytoplasmic envelopment, and interpreted as confirmation that the TGN acts as site of envelopment (Wang *et al.*, 2001).

The key contribution of VZV gE to cell-to-cell spread is seen not only in cell cultures, but also in an elegant in vivo model developed by the Arvin laboratory, consisting of T-cells or skin cell xenografts in the SCHID-hu mice system (Santos *et al.*, 2000). Of note, in the same system, gI is necessary, despite the fact that it is dispensable in cell culture (Moffat, *et al.*, 2002).

Recent studies with VZV gE have greatly expanded the role of this glycoprotein in replication in general and egress in particular. As part of a larger project to produce recombinant VZV genomes, several mutations were introduced in the cytoplasmic tail of gE, and the mutated gE was inserted back into an otherwise complete VZV genome (Moffat *et al.*, 2004). A single mutation in the YAGL endocytosis motif was lethal, whereas other mutations were not. Shortly thereafter, endocytosis of gE was documented to be an important trafficking mechanism for the delivery of this glycoprotein to the site of virion assembly in the cytoplasm of infected cells (Maresova *et al.*, 2005). Altogether the properties of gE, its role in fusion, as well as the location of its gene next to that of gD in HSV genome (discussed in Chapter 7) make VZV gE the analogue of HSV gD.

A third protein that plays a role in VZV egress is gB, particularly its endodomain. As is the case with gB from HSV and other herpesviruses, the endodomain contains determinants for the intracellular transport and localization of gB, including an ER to Golgi transport signal, and two tyrosine-based internalization signals for trafficking from the plasma membrane to the Golgi. Deletion of the portion of gB endodomain encoding these motifs alters gB localization at the Golgi apparatus and drastically reduces the transport of VZvirions to the extracellular space (Heineman and Hall, 2001, 2002). VZV gB endocytosis, as it relates to fusion activity, has been reviewed in Cole and Grose (2003), and dealt with in Chapter 7.

Thus, in addition to gE, endocytosis of gB and also that of gH have been documented to be a means for their delivery from the surface of the infected cells to the cytoplasmic site of virion assembly (Pasieka *et al.*, 2003, 2004). These studies were performed by first biotinylating cell surface proteins, and then demonstrating that they were subsequently transported to purified virions isolated from infected cells by density gradient sedimentation (Maresova *et al.*, 2004). The results were confirmed by immunolabeling and transmission electron microscopy. Cumulatively, these findings highlight that endocytosis must be added to the trafficking pathways by which glycoproteins can be transported to the cytoplasmic site of envelopment, as part of the de-envelopment–re-envelopment model.

It should be noted that PrV gE does not follow the same trafficking pathways as VZV gE. Also PrV gE is endocytosed.

However, trafficking studies with PrV gE demonstrated that little or no gE from the cell surface is subsequently carried to and incorporated within the virion (Tirabassi and Enquist, 1999). These differences may be related to the fact that gE is an essential glycoprotein in VZV, but not in PrV.

A peculiar morphological feature of VZV egress, not observed with HSV or other herpesviruses, is the exit from the infected cell surface in a distinctive pattern designated as"viral highways." By scanning electron microscopy, these consist of thousands of particles arranged in a linear pathway across the syncytial surfaces. gE is a determinant of this polarized egress (Santos *et al.*, 2000).

Concluding remarks

A growing support to the de-envelopment-re-envelopment pathway of alphaherpesviruses exit has been provided in recent years. In some cases, the evidence rests on conclusions that are not unique, and alternative interpretations of data are possible. As outlined by Enquist and collaborators a few years ago, "evidence supporting both models of herpesvirus exit can be found, and neither model has been disproved conclusively" (Enquist *et al.*, 1998). This remark still holds true.

We also note that most of the attention has been paid to elucidate the role of viral gene products in virus exocytosis, while the contribution of cellular proteins, and of the exocytic compartment in general, has been largely neglected. Studies of the role of cellular functions is complicated by the fact that the cell is deeply modified following HSV infection, e.g., the Golgi apparatus is fragmented into small, but functional pieces, the TGN is redistributed, the architecture of nuclear and cytoplasmic cytoskeleton are altered (Campadelli-Fiume *et al.*, 1993; Avitabile *et al.*, 1995; Scott and O'Hare, 2001; Reynolds *et al.*, 2004; Wisner and Johnson, 2004), phenomena which contribute to mislocalization of cellular markers. These changes are strongly cell line dependent, and may contribute, in part, to apparent discrepancies between different studies. Finally, it is worth noting that most of the studies on alphaherpesviruses' exocytosis have been carried out in cultures of epithelial cells or fibroblasts. Exit from the neuronal cells may well be different.

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13

The strategy of herpes simplex virus replication and takeover of the host cell

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Introduction

The fundamental mission of all viruses is to replicate and spread, and above all, to persist in the host environment to which they have become adapted. Viruses vary with respect to the mechanisms by which they attain their objectives. This variation is reflected not only in the basic mechanisms of viral entry into cells, synthesis of viral proteins, viral nuclei acid synthesis, virion assembly, and egress but also with respect to the basic strategies by which they preclude the enormous resources of the host cell and of the multicellular organism from totally blocking viral replication. The terminology used: "totally blocking" is appropriate; in essence the evolution of functions encoded in the viral genome reflects a fundamental accommodation between replication and spread as well as persistence in the human population. A replication and spread that kills the host will not permit the survival of the virus. The objective of this chapter is to examine the basic strategies evolved by HSV to replicate in its cellular environment.

Gene content, organization, and fundamental design of the viral genome

Several aspects of the structure, content and function of the viral genome are worthy of note. They are as follows.

(i) We do not know with any degree of certainty the exact number of transcriptional units or proteins encoded by the viral genome. The problem stems from several considerations. The initial enumeration of open reading frames (ORFs) excluded sequences that lacked a TATA box, a canonical methionine intiation codon, a suitable length, or that situated antisense to a known or readily demonstrable ORF. The current list of HSV sequences that are expressed (see section on gene content) includes RNAs that do not appear to encode proteins (e.g., Ori_S RNAs), TATA-less ORFs (e.g., $\gamma_1$34.5), and ORFs that are antisense to each other (e.g., ORF P and ORF O vs. $\gamma_1$34.5, U_L43.5 vs. U_L43, U_L27.5 vs. U_L27, U_L9.5 vs. U_L10).

(ii) Viral proteins can express multiple functions. The order of expression of these functions may be regulated by post-translational modification of the proteins or by interacting proteins present within various compartments of the cell. For example, the interaction of ICP22 with cellular proteins is determined by the status of post-translational phosphorylation by viral kinases (Leopardi *et al.*, 1997b; Purves and Roizman, 1992; Purves *et al.*, 1993). Post-translational modification of ICP0 is also required for its ubiquitin-ligase activities (Boutell *et al.*, 2002; Hagglund *et al.*, 2002) but is not necessary for other functions required for optimal replication of the virus such as its interactions with CoREST (Gu *et al.*, 2005).

A powerful mechanism for regulation of specific functions encoded in a single protein is the synthesis from an independent transcriptional unit of a truncated protein whose sequence is colinear with the carboxyl-terminal domain of the larger protein. The functions encoded by the truncated proteins could enhance (e.g., U_S1.5 vs. α22) (Carter and Roizman, 1996; Purves *et al.*, 1993) or inhibit (U_L8.5 vs. U_L9) (Baradaran *et al.*, 1994) the function of the larger protein.

(iii) There is little doubt that herpes simplex virions contain at least three proteins (U_S11, U_L47 and U_L49) that bind RNA from the infected cell and translocate it into the newly infected cell (Roller and Roizman, 1990; Sciortino *et al.*, 2002). Evidence has been reported that this RNA can be expressed (Sciortino *et al.*, 2002). A central issue is the impact of this RNA on the outcome of infection. A fundamental unanswered question is why HSV would carry RNA rather than more tegument proteins. One argument presented by Shenk (2002) is that proteins destined to

membranes or secretory pathway would have to be made *de novo* and cannot be brought as components of the tegument.

(iv) The presence and function of the inverted repeats has been a long-standing puzzle. The repeats are present in many, but not in all herpesvirus genomes. Mutants lacking the inverted repeats replicate well in cultured cells but tend to be avirulent in experimental animals. The loss of virulence has been attributed to the loss of one copy of the $\gamma_1$34.5 genes but in fact one copy of the $\gamma_1$34.5 gene is sufficient to block the shut off of protein synthesis by activated protein kinase R (PKR) (He *et al.*, 1997a).

(v) As detailed in the section on gene content, more than half of the transcriptional units contained in the viral genome are not essential for viral replication at least in cultured cells. The functions encoded by many of these genes are unknown. Preliminary studies suggest that some genes may play a role in viral maturation or spread. Those that have been studied in detail (e.g., ICP0, U_L41, $\gamma_1$34.5, U_S3, U_L47, etc.) as well as some of the essential genes (e.g., α27) appear to encode function designed to block host responses to infection. The key observation relevant to this section is that viruses lacking non-essential genes do not circulate in nature and that in most instances mutants lacking one or more of these genes tend to be either incapable of replication or are less virulent than wild-type viruses in experimental animals.

In essence, the functional content of the HSV genome is not fully understood. We do not know the total number of diverse functions encoded by the viral genome. What is readily apparent is that many of the known functions encoded by the virus are directed toward total control of the infected cell. Some of these functions are redundant: they are expressed by different proteins but ultimately have the same objective. Since the functions of many "dispensable" proteins are not yet identified, there may be additional anti-host functions yet to be discovered.

Mobilization of cellular proteins for enhanced replication of HSV

There are three examples that illustrate recruitment and post-translational modifications that benefit viral replication. Current wisdom is that, on discharge of the viral DNA into the nucleoplasm, it is confronted by a wide range of cellular proteins that assemble around it and, if allowed to prevail, are likely to either enable minimal transcription or silence it, and viral transcriptional factors (e.g., α-transinducing factor or VP16) that activate transcription and ultimately enable its replication. Recent studies from several laboratories indicated the presence of histones associated with viral DNA but it is unclear whether the histones are associated with a small fraction or the vast majority of the DNA (Poon *et al.*, 2003). One perennial candidate for restructuring of DNA protein complexes to enable efficient transcription is ICP0, a product of the α0 gene (for review, see Hagglund and Roizman, 2004). ICP0 is a promiscuous transactivator of genes introduced into cells by infection or transfection, that is presumably of genes associated with cellular proteins but which have not yet assumed a tight chromatin structure. In recent studies unmodified ICP0, i.e., ICP0 made before significant accumulation of viral protein kinases, interacts with CoREST/REST and HDAC1 complexes and disrupts the interaction of HDAC1 with CoREST/REST complex (Gu *et al.*, 2005). Ultimately, late viral functions promote the export of CoREST and HDAC1 to the cytoplasm. This function of ICP0 is consistent with the hypothesis that activation of transcription of the viral genome is essential for efficient expression of viral genes and for denuding the DNA from cellular proteins that impede transcription and ultimately replication of viral DNA.

The second example centers on the observation that, early in infection, HSV-1 U_L13 kinase mediates the hyperphosphorylation of the translation elongation factor EF-1δ, an event associated with more efficient protein synthesis (Kawaguchi *et al.*, 1998). The U_L13 protein kinase is highly conserved among members of all three subfamilies of herpesviruses and studies on representative members indicate that this function of the U_L13 orthologs is also conserved (Kawaguchi *et al.*, 1999).

The third example relates to the function of ICP22, a product of the α22 gene. Mutants lacking this gene grow poorly and synthesize suboptimal amounts of a subset of late (γ_2) proteins exemplified by the U_S11, U_L38 and U_L41 proteins in primary human cell cultures and in some non-human cell lines (Purves *et al.*, 1993). ICP22 is a multifunctional protein. The functions enabling optimal synthesis of the subset of late proteins map to the carboxyl-terminal domain of ICP22 (Ogle and Roizman, 1999). At least two functions of ICP22 may account for the optimal synthesis of this subset of late viral proteins. First, ICP22 and U_L13 stabilize and activate the cyclin-dependent kinase cdc2 but enable the degradation of its partners, cyclins A and B (Advani *et al.*, 2000). cdc2 acquires a new partner, the HSV-1 DNA polymerase accessory protein encoded by U_L42 ORF (Advani *et al.*, 2001). The cdc2-U_L42 complex binds topoisomerase IIα – all in an ICP22-dependent manner (Advani *et al.*, 2003). It has been postulated that the mRNA encoding the subset of late proteins is transcribed off progeny DNA contained in massive tangles of concatemeric DNA

and that the presence of the topoisomerase enables more efficient transcription of the DNA. A second function of ICP22 and U_L13 protein kinase reported initially by Spencer and colleagues is to mediate the post-translational modification of the carboxyl-terminal domain of RNA polymerase II (RNA POL II) (Long *et al.*, 1999; Rice *et al.*, 1994, 1995). The modified RNA POL II acquires an "intermediate electrophoretic mobility" (RNA POL IIi). Recent studies in this laboratory indicate ICP22 interacts with the U_S3 protein kinase to recruit cdk9, a cyclin-dependent kinase involved in transcription (Durand *et al.*, 2005). The cdk9–ICP22 complex can phosphorylate the carboxyl-terminal domain of RNA POL II in vitro. This interaction appears to be independent of the posttranslational modification of RNA POL IIi. The presumption is that these modifications of RNA POL II enable more efficient elongation in the course of mRNA synthesis.

These are but three examples of modification of cellular structures designed to enhance the synthesis of viral gene products. Additional modifications are likely to emerge.

The objectives and general strategy of anti-host functions

A cursory review of the anti-host functions expressed by HSV indicates that they fall into four categories. In essence HSV (i) selectively blocks the synthesis of new proteins, (ii) blocks the function of preexisting cellular proteins activated after infection, (iii) selectively degrades cellular proteins (iv) blocks signaling to the host immune system indicating that the cell is infected. These functions are also expressed by other alphaherpesviruses and to a lesser extent by members of the beta- and gamma- herpesviruses. The fundamental strategy of HSV as opposed to that of members of the beta- and gamma- herpesviruses is to encode proteins that bind and subvert the function of cellular proteins. Unlike beta- and gamma-herpesviruses, HSV does not make extensive use of cellular orthologs to reinforce, substitute or block the function of cellular genes. At most, HSV encodes a short amino acid sequence related to that of a cellular protein such as the one present at the carboxyl terminal of the $\gamma_1$34.5 protein. This sequence is homologous to that of the corresponding domain of the GADD34 gene (Mohr and Gluzman, 1996).

The activation of NF-κB

On the basis of viral efforts to suppress its activation, NF-κB may well be viewed as a nemesis of viral replication and spread. Activation of NF-κB results in the synthesis of cytokines and growth factors that would lead to a potentially undesirable host response to infection (for review, see Santoro *et al.*, 2003). In HSV-1 infected cells NF-κB is activated (Amici *et al.*, 2001, Patel *et al.*, 1998), although studies on just a few products induced by activated NF-κB suggest that synthesis of NF-κB-dependent proteins may be selective (Esclatine *et al.*, 2004a,c).

In human and mouse cells tested to date, stable activation of NF-κB requires activated PKR (Taddeo *et al.*, 2003b). This protein is resident in cells and is activated by interferon and double stranded RNAs (for review, see Williams, 2001). Activated PKR phosphorylates the α subunit of the translation initiation factor 2 (eIF-2α). In turn, eIF-2α-P shuts off protein synthesis. Activated PKR could be viewed as an ancient response to infection and viruses have evolved many and diverse strategies to block its activation. Indeed HSV-1 contains a late gene: U_S11 which, when expressed early, blocks activation of PKR (Cassady *et al.*, 1998). HSV has evolved a very different but puzzling strategy to deal with PKR. Instead of blocking its activation, HSV encodes a protein, $\gamma_1$34.5, which binds the protein phosphatase 1α and redirects it to dephosphorylate eIF-2α (He *et al.*, 1997a,b, 1998). This strategy makes little sense in light of the observation that HSV-1 yields are 10- to 50-fold higher in PKR−/− cells than in sibling PKR+/+ cells (Taddeo *et al.*, 2004a). HSV requires activated PKR for some of its functions. Several lines of evidence indicate that activation of NF-κB is tied to activation of PKR. Thus, NF-κB is not activated by wild-type virus in infected PKR−/− cells. The HSV-1 mutant virus R5104 in which U_S11 was converted from a γ_2 to an early gene replicates as well as the wild-type virus but does not induce NF-κB (Taddeo *et al.*, 2003b).

A central question is why NF-κB is induced. As indicated above, wild-type HSV-1 replicated better in PKR−/− cells than in the sibling, PKR+/+ cells. In contrast, the yields of wild-type virus were at least 10-fold lower in NF-κB knockout fibroblasts, i.e. p50−/−, p65−/− and p50/p65−/− cells than in wild-type cells derived from NF-κB+/+ siblings (Taddeo *et al.*, 2004a). Currently, two hypotheses prevail. One hypothesis holds that NF-κB induces the synthesis of proteins that are required to block apoptosis (Goodkin *et al.*, 2003; Gregory *et al.*, 2004). The other hypothesis is that HSV-1 requires NF-κB-dependent proteins for its replication. This laboratory failed to find support for the first hypothesis. Indeed, HSV-1 encodes at least three proteins known to block apoptosis (see below) and p50−/−, p65−/− and p50/p65−/− cells appear to be resistant to apoptosis induced by defective HSV-1 viruses, although they are sensitive to exogenous pro-apoptotic agents

(Taddeo *et al.*, 2004a). The second hypothesis remains unproven.

Degradation of mRNA in infected cells

Studies carried out several decades ago (Roizman *et al.*, 1965; Sydiskis and Roizman, 1966, 1968) showed that cellular protein synthesis is downregulated, but the mechanisms did not become apparent until Frenkel and associates (Kwong *et al.*, 1988; Strom and Frenkel, 1987) described the role of U_L41 protein, dubbed *vhs* for virion host shutoff, in degrading mRNA (for review, see Smiley, 2004). The typical experiment designed to show the degradation involved a short labeled amino acid pulse at a time after infection. The results of these experiments showed that incorporation of amino acids into both viral and cellular proteins was diminished in wild-type virus infected cells but not in ΔU_L41 mutant infected cells. Other studies demonstrated that both viral and cellular mRNAs were degraded but that viral RNA transcripts nevertheless accumulated in infected cells, possibly as a consequence of higher rates of transcription of viral genes.

Subsequent studies reported that U_L41 interacted with the translation elongation factors eIF-4H and eIF-4B to degrade mRNA beginning at or near its 5′ terminus (Doepker *et al.*, 2004; Everly *et al.*, 2002; Karr and Read, 1999; Perez-Parada *et al.*, 2004). Moreover, consistent with the observation that U_L41 is a γ_2 structural protein made late in infection and hence if active would preclude the synthesis of viral protein, it was shown that the degradation of RNA ablated several hours after infection very likely as a consequence of the interaction of U_L41 protein with VP16 encoded by the U_L48 ORF (Smibert *et al.*, 1994). Other studies demonstrated that the shut-off of cellular protein synthesis was also the consequence of the function of ICP27. Early in infection ICP27 blocks the splicing of mRNA (Hardwicke and Sandri-Goldin, 1994). Late in infection, defined in reference to the initiation of synthesis of viral DNA, ICP27 shuttles between the nucleus and cytoplasm (Sandri-Goldin, 1998). The anti-RNA splicing functions of ICP27 do not appear to affect viral gene expression since, of the four transcriptional units that yield spliced mRNAs, three (α0, α22, α47) are expressed before the effect of ICP27 becomes apparent whereas the fourth (U_L15) is expressed at a time when ICP27 shuttles RNA. The net effect of blocking splicing is that unspliced mRNA is transported into the cytoplasm.

More recent studies (Esclatine *et al.*, 2004a,c; Taddeo *et al.*, 2003a) indicate that the degradation of cellular mRNA is a consequence of a process far more complex than that deduced from earlier studies. In essence, the degradation of mRNA and the mechanism by which this degradation is achieved appears to vary. In the case of housekeeping and constitutively expressed mRNAs (e.g., GAPDH and β-actin) the mRNAs are rapidly degraded very early after infection by an unknown mechanism in a U_L41-dependent fashion (Esclatine *et al.*, 2004c). In contrast, several mRNAs induced after infection and which normally have a relatively short half-life appear to linger even though the translation products may or may not accumulate. For example, IEX-1 mRNA is induced after infection (Taddeo *et al.*, 2002, 2003b). This mRNA contains AU-rich pentameric elements (AREs) in its 3′ untranslated region (UTR) that signal a short half-life (Bakheet *et al.*, 2003; Chen and Shyu, 1995). In mock-infected cells subject to stress this mRNA is made and then sequestered in exosomes (for review, see Butler, 2002) by members of the tristetraprolin (TTP) family (Chen *et al.*, 2001) and rapidly degraded in a 3′ to 5′ direction (Wilusz *et al.*, 2001). IEX-1 mRNA induced in ΔU_L41-infected cells is subject to a similar rapid degradation process (Esclatine *et al.*, 2004c). Unlike the situation in mock-infected cells, the mRNA appears to be stable in wild-type virus infected cells (Esclatine *et al.*, 2004c). However, a more detailed examination of the mRNA accumulating in the cytoplasm of wild-type virus infected cells indicated that this mRNA consists of full-length processed mRNA, mRNA containing the single intron of IEX-1, and finally, truncated forms of the mRNAs (Esclatine *et al.*, 2004a; Taddeo *et al.*, 2003a). The bulk of full-length mRNAs appear to be deadenylated. The truncated forms arise by 3′ to 5′ degradation and endonucleolytic cleavage at or near AREs (Esclatine *et al.*, 2004a). It is thus not surprising that IEX-1 protein is not detected beyond the first hour after infection. A similar picture is presented by mRNAs encoding IκBα and c-*fos* (Esclatine *et al.*, 2004a). Although all of these mRNAs contain AREs, not all AREs-containing mRNAs are subject to a similar fate. Thus TTP mRNA, which contains AREs in the 3′ UTR (Brooks *et al.*, 2004), is upregulated and translated to yield relatively large amounts of protein (Esclatine *et al.*, 2004a,b; Taddeo *et al.*, 2004b). Another mRNA, that of GADD45β, is also upregulated and translated (Esclatine *et al.*, 2004a). This mRNA does not contain AREs.

The model that best takes into account the available data is that HSV attempts to counter the multiple pathways of activation of stress responses by insuring that they are fully activated (e.g., specific activation of NF-κB) and then blocking those that are inimical to viral replication. Among these are responses that signal to the environment that the cell is infected or that activate pro-apoptotic pathways. A key cellular gene activated in the course of infection and which may be the basis of discrimination between mRNAs that

are degraded and those that are not is TTP. As noted above, members of the TTP family regulate the life span of AREs mRNAs (for review, see Blackshear, 2002). As yet, preliminary studies indicate that U_L41 protein interacts with components of the exosome (Esclatine *et al.*, 2004b; B. Taddeo and B. Roizman, unpublished data). Of the many issues yet unresolved is the mechanism by which some mRNAs containing AREs (e.g., TTP mRNA) are less rapidly degraded than those encoding IEX-1 or the molecular basis for the U_L41-dependent rapid degradation of GAPDH or β-actin mRNAs as compared to those of mRNAs containing AREs (Esclatine *et al.*, 2004c).

Specific degradation of cellular proteins in wild-type virus-infected cells

It could be expected that proteins with a relatively short half-life and whose mRNA is degraded after infection, as described above, would disappear and not be replenished in the wild-type virus infected cells. An example of just such a protein is Jak1, described later in the text. Analyses of a relatively small number of cellular proteins led to the discovery of several proteins that turn over in infected cells in a manner dependent on α0 rather than on U_L41 and α27 genes. The cellular proteins degraded in an ICP0-dependent manner include the promyelocytic leukemia protein (PML) SP100, the centromeric proteins (CNP) A and C, and the DNA-dependent protein kinase. In contrast to the disappearance of the proteins listed above, cyclins D3 and D1 turned over at a much slower rate or were stabilized over many hours after infection (for review, see Hagglund and Roizman, 2004).

ICP0 is a 775-residue protein translated from a spliced mRNA. The coding sequences are derived from 3 exons containing 19, 241, and 515 residues, respectively. The protein contains a RING finger located between residues 116 and 156 of exon 2. The protein was shown to interact physically with a ubiquitin specific protease (USP7), EF-1δ, the transcriptional factor BMAL and a protein of unknown function designated as p60 (for review, see Hagglund and Roizman, 2004). Early in infection ICP0 localizes in nuclear structures known as PODs, ND10, etc. Within a few hours after infection, ICP0 mediates the degradation of ND10 structures and becomes dispersed throughout the cytoplasm. After the onset of viral DNA synthesis, ICP0 is translocated into the cytoplasm. The available data indicate that ICP0 is actually retained in the cytoplasm by a β or γ_1 function until viral DNA synthesis inasmuch as in cells infected with the d120 (Δα4) mutant, ICP0 is translocated to the cytoplasm and aggregated with proteasomes after the degradation of ND10 but within a very short time after infection (Lopez *et al.*, 2001).

Three lines of investigation led to the realization that ICP0 acts as a two-sited ubiquitin ligase (for review, see Hagglund and Roizman, 2004). First, ICP0 has many features similar to that of ubiquitin ligases. Thus the RING finger is characteristic of many ubiquitin ligases and ICP0 dynamically interacts with proteasomal components. Second, the dispersal of ND10 could be directly related to the ICP0 domain encoding the RING finger. The third line of evidence emerged from studies of the interaction of ICP0 with cyclins D3 and D1. Thus, it has been shown that ICP0 could interact physically and genetically with cyclin D3, but not with cyclin D1, and independently that cdc34 was degraded in infected cells in a proteasome- and ICP0-dependent manner. The observations that both proteins did not turn over as rapidly as expected and that no new transcripts were detected suggested that ICP0 blocks their turnover possibly by degrading the ubiquitin conjugating enzyme (cdc34 or UbcH3) responsible for the degradation of cyclin D1 in uninfected cells. In vitro substrate-independent assays showed that cdc34 was polyubiquitylated in an ATP-dependent manner by sequences encoded by exon 3 rather than the expected exon 2 (Hagglund and Roizman, 2002; Hagglund *et al.*, 2002). The ubiquitylation activity was mapped to residues 621 to 624 and indeed viral mutant carrying ICP0 gene lacking these sequences did not bind or mediate the degradation of cdc34 or the stabilization of the D cyclins (Hagglund and Roizman, 2002). In addition, RING domain mutants did not affect the degradation of cdc34. The ubiquitin conjugating enzymatic site mapping in the sequences encoded by exon 3 was designated herpesvirus ubiquitin ligase 1 (HUL-1), does not resemble in sequence any of the known ubiquitin ligases and may therefore represent a new class of these enzymes.

The in vitro substrate-independent ubiquitylation assays indicated that the sequences encoded by exon 2 polyubiquitylated UbcH5A and UbcH6 ubiquitin conjugating enzymes but not a large number of others. This observation indicated that exon 2 encodes a second ubiquitin conjugating enzyme (HUL-2) (for review, see Hagglund and Roizman, 2004) and further assays using dominant negative ubiquitin conjugating enzymes established that HUL-2 employs UbcH5A to degrade PML, SP100, and the DNA-dependent protein kinase (Gu and Roizman, 2003). The context of the interaction of ICP0 with UbcH6 or the mechanism by which ICP0 mediates the degradation of CENP-C and CENP-A are not known. It is conceivable that ICP0 targets other protein for degradation.

The objectives attained by the degradation of cdc34 and PML are at least in part apparent. By degrading cdc34,

HSV precludes the degradation of cyclin D3. This protein appears to mediate the transport of ICP0 to the ND10 structure and later to the cytoplasm. A mutation that affects the binding of ICP0 to cyclin D3 affects viral replication in stationary cells and reduces the viability of the virus administered at a peripheral site from invading the central nervous system (Hagglund and Roizman, 2003). Stabilization of cyclin D3 does not drive the cell into the S phase.

PML is the organizing protein of the ND10 structures. An extensive literature attributes to ND10 numerous functions related to transactivation or repression of cellular genes (for review, see Everett, 2001; Regad and Chelbi-Alix, 2001). Ectopic overexpression of PML did not affect viral replication, although it resulted in the formation of highly enlarged ND10 structures that were not dispersed in the course of HSV-1 infection (Lopez *et al.*, 2002). Similarly, wild-type virus replicated equally well in both PML−/− and PML+/+ cells. However, on pretreatment of cells with either interferon α or γ resulted in a significantly higher decrease in viral replication in PML+/+ cells as compared to PML−/− cells (Chee *et al.*, 2003). The necessary conclusion derived from these studies is that the activation of the antiviral state is mediated through ND10 and that the objective of degrading PML and dispersal of ND10 is to preclude the potential inhibition of viral replication by exogenous interferon.

Shut down of the interferon pathways to host resistance to infection

The preceding sections cited several mechanisms by which HSV blocks interferon-mediated pathways of host resistance to infection.

(i) Both $\gamma_1$34.5 and, to a lesser, degree U_S11 proteins circumvent the consequences of phosphorylatation of eIF-2α, the former by sequestering and redirecting phosphatase 1α to dephosphorylate eIF-2α (He *et al.*, 1997a,b, 1998). The effect on the interferon pathway is apparent from the observation that $\Delta\gamma_1$34.5 mutants are highly attenuated in wild-type mice but not in mice lacking interferon receptors (Leib *et al.*, 1999, 2000).

(ii) Some of the proteins in the interferon pathways have a relatively short half life (e.g., Jak1). The U_L41-dependent RNase activity blocks the replenishment of Jak1 and possibly other proteins in the pathway (Chee and Roizman, 2004).

(iii) As noted above, absence or overexpression of PML has no effect on viral replication. The role of PML and the objective of insuring its degradation are readily apparent in interferon-treated cells. In absence of PML, the infected cells are insensitive to either α- or γ-interferon (Chee *et al.*, 2003). Thus the interferon-based activation of antiviral genes is mediated via the ND10 structures. By targeting PML for degradation, HSV pre-empts antiviral activity of exogenously produced interferon.

Consistent with the overall strategy of viral interference with host defense mechanisms, the pathways by which the HSV blocks the interferon pathways of host defense are redundant but the above list may not be exhaustive. For example, in recent studies it has been observed that the cells surface receptor for interferon γ is rapidly reduced after infection (Y. Liang and B. Roizman, unpublished data). The mechanism by which HSV causes a reduction in the amount of this receptor is not yet known. It is conceivable that as yet other anti-interferon pathways are encoded by the viral genome.

HSV blocks pro-apoptotic cellular functions

Programmed cell death or apoptosis is a mechanism by which a multicellular organism rids itself of unwanted cells. External or internal stimuli activate a cascade of proteases that degrade essential proteins and ultimately the cellular DNA. In the context of infection, apoptosis is a supreme sacrifice by cells to diminish the impact of the infection and enable the survival of the organism. While earlier studies on other viruses and human cytomegalovirus (Shen and Shenk, 1995; Zhu *et al.*, 1995) anticipated that HSV-1 would regulate apoptosis, three fundamental reports initiated an exciting excursion into the control of pro-apoptotic pathways by HSV. Thus, within a short time, three laboratories reported that HSV blocked apoptosis induced by FAS ligand (Galvan and Roizman, 1998; Jerome *et al.*, 1999, 2001; Sieg *et al.*, 1996), osmotic shock, thermal shock (Galvan and Roizman, 1998; Galvan *et al.*, 1999; Koyama and Miwa, 1997; Leopardi and Roizman, 1996), and a defective viral mutant lacking the genes encoding ICP4 (Galvan and Roizman, 1998; Sieg *et al.*, 1996; Munger *et al.*, 2001). Subsequent studies identified at least two other viral mutants capable of inducing apoptosis and at least four genes whose products block programmed cell death induced by viral gene products or by a variety of exogenous agents (Aubert and Blaho, 1999; Benetti *et al.*, 2003; Jerome *et al.*, 1999, 2001; Leopardi *et al.*, 1997a; Munger and Roizman 2001; Munger *et al.*, 2001; Murata *et al.*, 2002; Perkins *et al.*, 2002; Zhou and Roizman, 2001; Zhou *et al.*, 2000). The key issues are the mechanisms by which viral or exogenous biologic agents induced programmed cell death and the mechanisms by which the virus blocks

apoptosis. The current literature may be summarized as follows.

(i) Wild-type HSV-1 does not induce apoptosis in cultured cells in the course of productive infection. Viral mutants reported to induce apoptosis are those lacking α4 (Galvan and Roizman, 1998; Leopardi and Roizman, 1996; Munger *et al.*, 2001), α27 (Aubert and Blaho, 1999) or U_L39, the gene encoding the major subunit of ribonucleotide reductase (Perkins *et al.*, 2002). The mechanism by which these mutants induce apoptosis is not clear. There is no compelling evidence to support the argument that all viral mutants induce apoptosis by a similar mechanism. For example, the mutant lacking the α4 genes induces apoptosis in virtually all cell lines tested to date including Vero cells, whereas the mutant lacking the α27 gene induces apoptosis in some cell lines but not in Vero cells (Aubert and Blaho, 1999). Overexpression of ICP22, ICP0 or ICP27 did not lead to apoptosis in several of the cell lines tested.

(ii) Of the four viral proteins shown to block apoptosis, i.e., ribonucleotide reductase, the U_S3 protein kinase, and the glycoproteins D and J (gD and gJ), only three have been studied in some detail. Nothing is known of the mechanism of action of the U_L39 gene product other than the observation that ΔU_L39 mutants complemented by U_L39 protein do not induce apoptosis (Perkins *et al.*, 2002). It should be noted that the HSV-2 U_L39 protein encodes a kinase but the substrates of the kinase other than itself are not known.

(iii) Ectopic expression of the U_S3 protein kinase was initially reported to block apoptosis induced by the Δα4 mutant (Leopardi and Roizman, 1996). Subsequently, this protein was shown to block apoptosis induced by a variety of agents including sorbitol, ectopic expression of several pro-apoptotic genes including BAD, Bax, Bid (Benetti *et al.*, 2003; Munger and Roizman, 2001; Ogg *et al.*, 2004). Curiously, U_S3 does not appear to phosphorylate BAD contrary to the published report inasmuch as BAD protein lacking the known phosphorylation sites induces apoptosis that is blocked by the U_S3 protein kinase (Benetti *et al.*, 2003). ΔU_S3 mutants induce apoptosis albeit weakly and only in some cell lines (e.g., rabbit skin cells) but not in human or primate cells susceptible to apoptosis induction by other viral mutants. More recent studies have shown that the substrate specificity of U_S3 protein kinase is very similar to that of cyclic AMP dependent protein kinase A (PKA) (Benetti and Roizman, 2003). Thus forskolin, an inducer of PKA, blocks apoptosis induced by the Δα4 mutants, and that a specific inhibitory peptide that blocks activation of PKA reverses the effect of forskolin. U_S3 also mediates the phosphorylation of the regulatory subunit of PKA in in vitro studies. Still more recent studies place U_S3 in mitochondria (Van Minnebruggen *et al.*, 2003). The data suggest that the anti-apoptotic substrates of U_S3 protein kinase are mitochondrial proteins and that activated PKA may substitute for the U_S3 protein kinase in blocking apoptosis.

(iv) The role of gD and gJ in blocking apoptosis emerged from the observation that viral mutant stocks lacking gD in both the gene and the protein in the virion envelope (gD−/−) or viral stocks lacking the gD gene but containing the glycoprotein provided by cells ectopically expressing the protein (gD−/+) induced apoptosis that was blocked by ectopic expression of gD or gJ (Zhou and Roizman, 2001; Zhou *et al.*, 2000). The mechanisms by which the two stocks induce apoptosis differ. The model that best fits the results of the studies on gD as the blocker of apoptosis is as follows. In cells exposed to high rations of gD−/− mutant per cell, the virus enters the cells by endocytosis and triggers massive lysosomal discharge that causes cell death. Consistent with these events, chloroquine blocks apoptosis induced by gD−/− mutants (Zhou and Roizman, 2002a). To block apoptosis, gD must be either intact or consist of ectodomain and transmembrane domain linked by disulfate bonds to a transmembrane domain and a cytoplasmic domain (Zhou and Roizman, 2002b). In contrast, gD−/+ mutant stocks induce apoptosis even after infection of cells at low multiplicities of infection, chloroquine does not block apoptosis and the evidence suggests that apoptosis is induced late rather than early in infection. In this instance the etcodomain of gD is the only component of gD essential for blocking apoptosis induced by gD−/+ stocks. One hypothesis that remains to be explored in greater detail rests on an earlier report that gD interacts and colocalizes with the cation-independent mannose-6-phosphate receptor, a regulator of lysosomal enzyme transport (Zhou and Roizman, 2002a). The hypothesis predicts that, to block apoptosis induced by gD−/− mutant, the ectopically expressed gD must be translocated to the lysosomal vesicles and interact with the mannose-6-phosphate receptor. In contrast, the gD−/+ virus does not trigger apoptosis by lysosomal discharge but rather infects cells, replicates, and it is the gD−/− progeny of the gD−/+ virus that triggers apoposis during its transport through the exocytic pathway. The target of gD in this instance could be mannose-6-phosphate receptor or another as yet unidentified protein.

(v) gJ is the smallest of the glycoproteins encoded by HSV and its function until recently was largely unknown. Studies by Jerome and colleagues indicate that gJ blocks Fas ligand- or granzyme B-induced apoptosis and more important, it is involved in the protection of infected cells from CTL-induced apoptosis (Jerome *et al.*, 1998, 2001).

Conclusions

As indicated earlier in the text, the strategic objective underlying the evolution of HSV is total control of the biosynthetic and defensive pathway of the cell. This is achieved though physical interaction between viral and cellular proteins and results in the modification and diversion of the host protein to fulfill the needs of the virus. The three fundamental conclusions of the studies carried out so far are that (i) viral proteins are largely multifunctional, the function at any given time most likely determined either by the partner to which they are bound or the post-translational modification to which they are subjected; (ii) the full complement of viral protein functions remains to be discovered and (iii) HSV exhibits a functional but not sequence driven redundancy. In essence, numerous functions expressed by different viral proteins aim to modify or suppress a specific metabolic pathway, although the targeted aspects of the pathway are not identical. If HSV had the arrogance (and capacity) to express it, its motto would be "we take no chances."

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Part II

Basic virology and viral gene effects on host cell functions: betaherpesviruses

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14

Comparative genome and virion structure

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Introduction

The two major lineages in the *Betaherpesvirinae* are the cytomegaloviruses (the *Cytomegalovirus* and *Muromegalovirus* genera, plus a number of other viruses whose taxonomy is only partially defined) and the *Roseolovirus* genus (see Chapter 1). The best characterized members of these lineages are HCMV (the prototype of the subfamily) and HHV-6, respectively. Cytomegaloviruses are present in a wide range of mammalian species, and have been termed "salivary gland viruses" because of their ease of isolation from explanted tissue. An earlier divergence of the *Betaherpesvirinae* may be represented by a herpesvirus of elephants (Richman *et al.*, 1999; Ehlers *et al.*, 2001). This chapter starts by describing the genome structures of *Betaherpesvirinae*, then examines the genetic content of HCMV and HHV-6, and finally focuses on the virion structure of HCMV.

Genome structures

The genomes of viruses in the *Roseolovirus* genus are significantly smaller, at 145–162 kbp, than those of other *Betaherpesvirinae*, at 196–241 kbp. Indeed, HCMV has the largest genome among the human herpesviruses, and thus far its closest relative, CCMV, has the largest genome of all sequenced herpesviruses. It seems likely that the ancestor of the *Betaherpesvirinae* had a genome consisting of a unique region flanked by a direct repeat (the class A genome described in Chapter 2), since this structure is characteristic of most extant members of the subfamily. Earlier studies ruled out the presence of large repeats in the genomes of MCMV (Ebeling *et al.*, 1983a; Mercer *et al.*, 1983; Marks and Spector, 1984), THV (Koch *et al.*, 1985) and RCMV (Meijer *et al.*, 1986). Later work confirmed that MCMV and RCMV have terminal direct repeats of 30 and 504 bp, respectively (Marks and Spector, 1988; Vink *et al.*, 1996). A corresponding repeat has not been detected in THV, but the genome termini have not been examined directly. GPCMV has a terminal direct repeat of about 1 kbp, and a proportion of genomes lacks one copy (Gao and Isom, 1984; McVoy *et al.*, 1997; Nixon and McVoy, 2002). RhCMV has a terminal repeat of about 500 bp that may be present in multiple copies in some genomes (Hansen *et al.*, 2003). Interestingly, this resembles the class B genome structure found in many *Gammaherpesvirinae*. Information on the genome of another monkey cytomegalovirus, SCMV, is also available (Huang *et al.*, 1978; Jeang and Hayward, 1983), but the termini have not been characterized. The most commonly studied strain of SCMV, Colburn, was isolated ostensibly from a human (Huang *et al.*, 1978). Sequences from an agent dubbed "stealth virus," which was also suggested to be of human origin, have shown that this virus is SCMV (Martin, 1999).

Cytomegaloviruses of higher primates, HCMV and CCMV, have the class E genome structure, in which two unique regions (U_L and U_S) are flanked by direct repeats (TR_L and IR_L; TR_S and IR_S) (Weststrate *et al.*, 1980; Davison *et al.*, 2003a). A copy of the terminal direct repeat of approximately 300–600 bp (the *a* sequence) is present in inverted orientation at the junction between IR_L and IR_S (Spaete and Mocarski, 1985; Tamashiro and Spector, 1986). The genomes exhibit segment inversion, which results in virions containing equimolar amounts of four genome isomers differing in the relative orientations of U_L and U_S. The genomes of two closely related herpesviruses of New World monkeys (aotine herpesviruses 1 and 3) appear to have a similar structure (Ebeling *et al.*, 1983b). However, as mentioned above, RhCMV, a virus of an Old World monkey, does not. This suggests that the class E genome structure arose in an ancestral primate cytomegalovirus from a class

A genome by duplication of fused terminal sequences at a location internally in the genome, but that this duplication was lost in the lineage represented by RhCMV. The biological reason for a class E genome structure, which is also characteristic of certain alphaherpesviruses such as HSV-1 and HSV-2 (see Chapter 5), remains unknown.

The genomes of both variants of HHV-6 (referred to as HHV-6A and HHV-6B; Martin *et al.*, 1991b; Lindquester and Pellett, 1991) and HHV-7 (Dominguez *et al.*, 1996) contain a substantial terminal direct repeat, whose size was determined by sequencing as 8.1–8.8 kbp in the former and 5.8–10.0 kbp in the latter. The ranges are due to the presence in the direct repeat of regions of variable size consisting of reiterated short sequences.

Genes

Genome sequences

Complete genome sequences are available for eight species in the *Betaherpesvirinae*, as listed in Table 2.1. These include HCMV (Chee *et al.*, 1990; Murphy *et al.*, 2003b; Dunn *et al.*, 2003b; Dolan *et al.*, 2004), MCMV (Rawlinson *et al.*, 1996), RCMV (Vink *et al.*, 2000), THV (Bahr and Darai, 2001), RhCMV (Hansen *et al.*, 2003), CCMV (Davison *et al.*, 2003a), HHV-6A (Gompels *et al.*, 1995), HHV-6B (Dominguez *et al.*, 1999; Isegawa *et al.*, 1999) and HHV-7 (Nicholas, 1996; Megaw *et al.*, 1998). Partial sequence data are also available in the literature or public sequence databases for *Betaherpesvirinae* that infect Old World primates, such as the baboon (Blewett *et al.*, 2001), drill (Blewett *et al.*, 2003), colobus monkey and orangutan, the New World primate, aotine herpesvirus 1, and members of the *Suidae* including the domestic pig (Ehlers *et al.*, 1999; Rupasinghe *et al.*, 2001; Widen *et al.*, 2001) and the warthog (Ehlers and Lowden, 2004).

Genetic content

The remainder of this section focuses on a genetic comparison of HCMV, as the most extensively characterized cytomegalovirus, with HHV-6, in the *Roseolovirus* genus. Derivation of the genetic content of HCMV has been a protracted process, because strain AD169, which was sequenced first (Chee *et al.*, 1990), lacks 15 kbp at the right end of U_L in comparison with wild-type strains (Cha *et al.*, 1996), having in place of the deleted sequence an inverted duplication of a 10 kbp region from the left end of U_L. AD169 also bears several additional mutations (Davison *et al.*, 2003a) that can vary with respect to the strain variant being used and have important functional consequences (Skaletskaya *et al.*, 2001). Also, all passaged strains examined to date are compromised in one or more genes by mutations that introduce deletions, frameshifts or termination codons (Akter *et al.*, 2003; Dolan *et al.*, 2004). The deduced gene layout in wild-type HCMV is shown in Fig. 14.1, and that of HHV-6 is shown in Fig. 14.2. The genetic content of HHV-6B appears to be the same as that of HHV-6A; nine additional small ORFs were listed by Dominguez *et al.* (1999) as unique to HHV-6B, but their protein-coding status remains unknown. HCMV and HHV-6 are currently estimated to contain 165 and 86 genes, respectively, counting duplicated genes only once. The gene content of HHV-7 is similar to that of HHV-6: all HHV-7 genes but one (U55B, which is related to U55) have HHV-6 counterparts, and all but three HHV-6 genes (U22, U83 and U94) have HHV-7 counterparts. The HHV-6 genome contains several regions consisting of complex and simple repeats (T1, T2, R1, R2 and R3 in Fig. 14.3), only one which encodes protein. In contrast, HCMV lacks such repeats, but many proteins contain quasi-repetitive regions of one or a few amino acid residues. Although infrequent in all herpesviruses, mRNA splicing is involved in transcription of at least 12 HCMV genes (7% of the total) and at least 14 HHV-6 genes (16%).

Table 14.1 details the correspondence between HCMV and HHV-6 genes, alongside functional information. HCMV and HHV-6, respectively, possess 40 and 41 of the 43 core genes inherited by the *Alpha-*, *Beta-* and *Gammaherpesvirinae* from their common ancestor (see Chapter 2), located centrally in the genomes. Both lack homologues to the genes encoding thymidine kinase and the small subunit of ribonucleotide reductase, and HCMV lacks the core gene encoding a homologue of a protein that binds to ori_{Lyt}, the origin of DNA synthesis used during productive replication. In addition, four genes common to HCMV and HHV-6 have clear homologues in the *Gamma-* but not the *Alphaherpesvirinae*. These are UL49 (related to BFRF2 and ORF66 in the *Lymphocryptovirus* and *Rhadinovirus* genera, respectively), UL79 (BVLF1 and ORF18), UL87 (BcRF1 and ORF24) and UL92 (BDLF4 and ORF31). A fifth gene, UL88, is distantly related to BTRF1 and ORF23 and positionally equivalent to a gene in the *Alphaherpesvirinae* (UL21 in HSV-1). A sixth gene, UL91, is positionally equivalent to BDLF3.5 and ORF30 in the *Lymphocryptovirus* and *Rhadinovirus* genera, respectively. These six genes are considered at present to have arisen after the lineage that led to the *Beta-* and *Gammaherpesvirinae* diverged from the *Alphaherpesvirinae*, but it is also possible that the ancestral core set included UL88 along with one or more of the others, which were lost at an early stage from the lineage giving rise to *Alphaherpesvirinae*.

Table 14.1. Proteins encoded by HCMV and HHV-6 genes

HCMV[a]	HHV-6[a]	Function[b]	Selected references[c]
RL1			
RL5A		RL11 family	Davison *et al.* (2003b)
RL6		RL11 family	Davison *et al.* (2003b)
RL10		Virion envelope glycoprotein	Spaderna *et al.* (2002)
RL11		RL11 family; IgG Fc-binding membrane glycoprotein	Lilley *et al.* (2001); Atalay *et al.* (2002)
RL12		RL11 family; putative membrane glycoprotein	
RL13		RL11 family; putative membrane glycoprotein	Yu *et al.* (2002)
UL1		RL11 family; putative membrane glycoprotein	
UL2		Putative membrane protein	
UL4		RL11 family; virion glycoprotein	Chang *et al.* (1989)
UL5		RL11 family; putative membrane protein	
UL6		RL11 family; putative membrane glycoprotein	
UL7		RL11 family; putative membrane glycoprotein	
UL8		RL11 family; putative membrane glycoprotein	
UL9		RL11 family; putative membrane glycoprotein	
UL10		RL11 family; putative membrane glycoprotein	
UL11		RL11 family; membrane glycoprotein	Hitomi *et al.* (1997)
UL13		Putative secreted protein	
UL14		UL14 family; putative membrane glycoprotein	
UL15A		Putative membrane protein	
UL16		Membrane glycoprotein; inhibits NK cell cytotoxicity	Kaye *et al.* (1992a); Cosman *et al.* (2001); Odeberg *et al.* (2003); Dunn *et al.* (2003b)
UL17			
UL18		UL18 family; putative membrane glycoprotein; MHC-I homologue; possibly inhibits NK cell cytotoxicity	Browne *et al.* (1990); Cosman *et al.* (1997)
UL19			
UL20		Putative membrane glycoprotein	
UL21A			
UL22A		Putative secreted glycoprotein	Rawlinson and Barrell (1993)
UL23	U2	US22 family; tegument protein	Adair *et al.* (2002)
UL24	U3	US22 family; tegument protein	Adair *et al.* (2002); [Mori *et al.* (1998)]
UL25		UL25 family; tegument phosphoprotein	Baldick and Shenk (1996)
UL26		US22 family; tegument protein; transcriptional activator of major immediate early promoter	Baldick and Shenk (1996); Stamminger *et al.* (2002)
UL27	U4, U7	Locus of resistance to maribavir	Komazin *et al.* (2003); Chou *et al.* (2004)
UL28	U7	US22 family (spliced to an unidentified upstream exon)	
UL29	U8	US22 family	
UL30			
UL31	U10	DURP family	Davison and Stow (2005)
UL32	U11	Major tegument phosphoglycoprotein (pp150); highly immunogenic; binds to capsids	Jahn *et al.*, (1987); Benko *et al.* (1988); Baxter and Gibson (2001); [Neipel *et al.* (1992)]
UL33	U12	GPCR family; membrane protein; putative chemokine receptor; virion protein	Margulies *et al.* (1996); Fraile-Ramos *et al.* (2002); [Isegawa *et al.* (1998)]
UL34		Represses US3 transcription	LaPierre *et al.* (2001)
	U13		
UL35	U14	UL25 family; tegument phosphoprotein; interacts with UL82 protein	Liu and Biegalke (2002); Schierling *et al.* (2004); [Stefan *et al.* (1997)]
	U15		
UL36	U17	US22 family; immediate early tegument protein; inhibitor of caspase-8-induced apoptosis (vICA)	Smith and Pari (1995b); Patterson and Shenk (1999); Skaletskaya *et al.* (2001)
UL37	U18	Immediate early envelope glycoprotein; possible auxiliary role in DNA replication; exon 1 product is mitochondrial inhibitor of apoptosis (vMIA)	Smith and Pari (1995b); Sarisky and Hayward (1996); Al-Barazi and Colberg-Poley (1996); Goldmacher *et al.* (1999)
UL38	U19		

(*cont.*)

Table 14.1. (*cont.*)

HCMV[a]	HHV-6[a]	Function[b]	Selected references[c]
UL40		Putative membrane glycoprotein; inhibits NK cell cytotoxicity	Tomasec *et al.* (2000)
	U20	Putative membrane glycoprotein	
	U21	Putative membrane glycoprotein	
	U22	Putative membrane glycoprotein	
	U23	Putative membrane glycoprotein	
	U24	Putative membrane protein	
	U24A	Putative membrane protein	
UL41A		Putative membrane protein	Dargan *et al.* (1997)
UL42		Putative membrane protein	Dargan *et al.* (1997); Mocarski *et al.* (1997)
UL43	U25	US22 family; tegument protein	Adair *et al.* (2002)
	U26	Putative multiple transmembrane protein	
UL44	U27	Processivity subunit of DNA polymerase (ICP36)	Mocarski *et al.* (1985); Ertl and Powell (1992); Weiland *et al.* (1994); Appleton *et al.* (2004); [Agulnick *et al.* (1993); Lin and Ricciardi (1998)]
UL45	U28	Large subunit of ribonucleotide reductase; lacks catalytic residues and is probably enzymatically inactive; tegument protein	McGeoch and Davison (1999); Patrone *et al.* (2003); [Sun and Conner (1999)]
UL46	U29	Component of intercapsomeric triplexes in capsids (mC-BP)	Gibson *et al.* (1996a)
UL47	U30	Tegument protein; possible role in intracellular transport; binds to UL48 protein	Baldick and Shenk (1996); Bechtel and Shenk (2002)
UL48	U31	High molecular weight tegument protein; binds to UL47 protein	Bradshaw *et al.* (1994); Gibson (1996); Ogawa-Goto *et al.* (2002)
UL48A	U32	Located on tips of hexons in capsids (SCP)	Gibson *et al.* (1996b); Baldick and Shenk (1996); Yu *et al.* (2005)
UL49	U33	Conserved in *Gammaherpesvirinae*	
UL50	U34	Inner nuclear membrane protein; role in egress of capsids from nucleus	Muranyi *et al.* (2002)
UL51	U35	Role in DNA packaging	
UL52	U36	Role in DNA packaging	
UL53	U37	Nuclear matrix protein; tegument protein; role in egress of capsids from nucleus	Muranyi *et al.* (2002); Dal Monte *et al.* (2002)
UL54	U38	Catalytic subunit of DNA polymerase; inhibited by pyrophosphate (e.g. foscarnet), nucleoside (e.g. ganciclovir) and certain non-nucleoside compounds	Heilbronn *et al.* (1987); Kouzarides *et al.* (1987); D'Aquila *et al.* (1989); [Teo *et al.* (1991)]
UL55	U39	Virion glycoprotein B (gB); component of gCI; involved in virus entry	Cranage *et al.* (1986); Boyle and Compton (1998); Boehme *et al.* (2004); [Ellinger *et al.* (1993)]
UL56	U40	Putative subunit of terminase; virion protein; binds to DNA packaging motif and exhibits nuclease activity; involved in inhibition by benzimidazole ribonucleosides and certain non-nucleoside compounds	Bogner *et al.* (1993); Bogner *et al.* (1998); Bradshaw *et al.* (1994); Krosky *et al.* (1998); Buerger *et al.* (2001); Scheffczik *et al.* (2002)
UL57	U41	Single-stranded DNA-binding protein	Kemble *et al.* (1987); Anders and Gibson (1988)
UL69	U42	Regulatory protein; tegument protein; contributes to cell cycle block; exhibits nucleocytoplasmic shuttling	Winkler *et al.* (1994); Winkler and Stamminger (1996); Hayashi *et al.* (2000); Lischka *et al.* (2001)
UL70	U43	Component of DNA helicase-primase complex; primase	McMahon and Anders (2002)
UL71	U44	Putative tegument protein	
UL72	U45	DURP family; derived from deoxyuridine triphosphatase; lacks catalytic residues and is enzymatically inactive	McGeoch and Davison (1999); McGeehan *et al.* (2001); Caposio *et al.* (2004); Davison and Stow (2005)
UL73	U46	Virion glycoprotein N (gN); component of gCII	Mach *et al.* (2000)
UL74	U47	Virion glycoprotein O (gO); component of gCIII	Huber and Compton (1997); Li *et al.* (1997); Huber and Compton (1998)
UL75	U48	Virion glycoprotein H (gH); component of gCIII; involved in entry	Cranage *et al.* (1988); Milne *et al.* (1998); [Liu *et al.* (1993); Mori *et al.* (2003b); Santoro *et al.* (2003)]
UL76	U49	Virion-associated regulatory protein	Wang *et al.* (2000); Wang *et al.* (2004)
UL77	U50	Role in DNA packaging	
UL78	U51	GPCR family; putative chemokine receptor	[Milne *et al.* (2000)]

Table 14.1. (*cont.*)

HCMV[a]	HHV-6[a]	Function[b]	Selected references[c]
UL79	U52	Conserved in *Gammaherpesvirinae*	
UL80	U53	Protease (N terminus) and minor capsid scaffold protein (C terminus)	Welch *et al.* (1991); Welch *et al.* (1993); Chen *et al.* (1996); Tong *et al.* (1996); Qiu *et al.* (1996); Shieh *et al.* (1996); [(Tigue *et al.* (1996)]
UL80.5	U53.5	Major capsid scaffold protein	Robson and Gibson (1989); Wood *et al.* (1997); Oien *et al.* (1997)
UL82	U54	DURP family; tegument phosphoprotein (pp71; upper matrix protein); transcriptional activator; targeted to ND10; targets Rb proteins for ubiquitin-independent proteosomal degradation	Ruger *et al.* (1987); Liu and Stinski (1992); Homer *et al.* (1999); Bresnahan and Shenk (2000b); Hofmann *et al.* (2002); Ishov *et al.* (2002); Kalejta and Shenk (2003); Davison and Stow (2005)
UL83	U55	DURP family; tegument phosphoprotein (pp65; lower matrix protein); suppresses interferon response	Ruger *et al.* (1987); Browne and Shenk (2003); Davison and Stow (2005)
UL84		DURP family; role in DNA replication; exhibits nucleocytoplasmic shuttling; binds to IE2 protein; transdominant inhibitor of IE transcription	He *et al.* (1992); Sarisky and Hayward (1996); Gebert *et al.* (1997); Lischka *et al.* (2003); Reid *et al.* (2003); Xu *et al.* (2004); Davison and Stow (2005)
UL85	U56	Component of intercapsomeric triplexes in capsids (mCP)	Baldick and Shenk (1996)
UL86	U57	Major capsid protein; component of hexons and pentons (MCP)	Chee *et al.* (1989); [Littler *et al.* (1990)]
UL87	U58	Conserved in *Gammaherpesvirinae*	
UL88	U59	Conserved in *Gammaherpesvirinae*; tegument protein	Baldick and Shenk (1996)
UL89	U66	Putative ATPase subunit of terminase; involved in inhibition by benzimidazole ribonucleosides and certain non-nucleoside compounds	Krosky *et al.* (1998); Buerger *et al.* (2001); Scheffczik *et al.* (2002)
UL91	U62	Positionally conserved in *Gammaherpesvirinae*	
UL92	U63	Conserved in *Gammaherpesvirinae*	
UL93	U64	Role in DNA packaging; tegument protein	
UL94	U65	Tegument protein; binds single-stranded DNA	Wing *et al.* (1996)
UL95	U67	Positionally conserved in *Gammaherpesvirinae*	
UL96	U68	Tegument protein	
UL97	U69	Serine-threonine protein kinase; tegument protein; phosphorylates ganciclovir; inhibited by maribavir; roles in DNA synthesis, DNA packaging and nuclear egress; phosphorylates UL44 protein	Littler *et al.* (1992); Sullivan *et al.* (1992); van Zeijl *et al.* (1997); Talarico *et al.* (1999); Wolf *et al.* (2001); Biron *et al.* (2002); Krosky *et al.* (2003a); Krosky *et al.* (2003b); Marschall *et al.* (2003); [Ansari and Emery (1999)]
UL98	U70	Deoxyribonuclease	Sheaffer *et al.* (1997); Gao *et al.* (1998)
UL99	U71	Myristylated tegument phosphoprotein (pp28)	Landini *et al.* (1987); Meyer *et al.* (1988); Sanchez *et al.* (2000)
UL100	U72	Virion envelope glycoprotein M (gM); component of gCII	Lehner *et al.* (1989); Kari *et al.* (1994)
	U73	Binds to origin of DNA synthesis; helicase	Inoue *et al.* (1994); Inoue and Pellett (1995)
UL102	U74	Component of DNA helicase-primase complex	Smith and Pari (1995a); McMahon and Anders (2002)
UL103	U75	Tegument protein	
UL104	U76	Portal protein; possibly interacts with terminase	Komazin *et al.* (2004); Dittmer and Bogner (2005)
UL105	U77	Component of DNA helicase-primase complex; helicase	Smith *et al.* (1996); McMahon and Anders (2002)
UL111A		Viral interleukin 10 (vIL-10)	Kotenko *et al.* (2000); Lockridge *et al.* (2000); Jones *et al.* (2002)
UL112	U79	Role in transcriptional activation or orchestrating DNA replication proteins	Penfold and Mocarski (1997); Ahn *et al.* (1999); Li *et al.* (1999)
UL114	U81	Uracil-DNA glycosylase; roles in excision of uracil from DNA and temporal regulation of DNA replication	Prichard *et al.* (1996); Courcelle *et al.* (2001)
UL115	U82	Virion envelope glycoprotein L (gL); component of gCIII; involved in entry	Kaye *et al.* (1992b); Milne *et al.* (1998); [Liu *et al.* (1993)]
UL116		Putative membrane glycoprotein	
	U83	CC chemokine	Zou *et al.* (1999); French *et al.* (1999); Lüttichau *et al.* (2003)

(*cont.*)

Table 14.1. (*cont.*)

HCMV[a]	HHV-6[a]	Function[b]	Selected references[c]
UL117	U84		
UL119	U85	IgG Fc-binding membrane glycoprotein related to OX-2	Atalay *et al.* (2002)
UL120		UL120 family; putative membrane glycoprotein	
UL121		UL120 family; putative membrane glycoprotein	
UL122	U86	Immediate early transcriptional activator (IE2); interacts with basal transcriptional machinery and cellular transcription factors; specific DNA-binding protein	Hermiston *et al.* (1987); Hagemeier *et al.* (1992); Arlt *et al.* (1994); Marchini *et al.* (2001); Heider *et al.* (2002); [Papanikolaou *et al.* (2002); Gravel *et al.* (2003)]
UL123	U90[d]	Immediate early transcriptional activator (IE1); enhances activation by IE2; interacts with basal transcriptional machinery and a cellular transcription factor; disrupts ND10	Hermiston *et al.* (1987); Greaves and Mocarski (1998); Ahn and Hayward (2000); Xu *et al.* (2001); Gawn and Greaves (2002); Lee *et al.* (2004); Nevels *et al.* (2004); [Martin *et al.*, 1991a); Gravel *et al.* (2002); Nikolauo *et al.* (2003)]
UL124	U91[d]	Putative membrane glycoprotein	
	U94	Parvovirus Rep protein homologue; binds to a transcription factor and single-stranded DNA; possible transcriptional regulator and involvement in latency	Thomson *et al.* (1991); Rotola *et al.* (1998); Mori *et al.* (2000); Dhepakson *et al.* (2002)
	U95	US22 family; immediate early gene	Takemoto *et al.* (2001)
	U100	Virion glycoprotein Q; complexed with gH and gL	Pfeiffer *et al.* (1995); Mori *et al.* (2003a)
	DR1	US22 family	
	DR6	US22 family; possible transactivator	Kashanchi *et al.* (1997)
UL128		Virion protein with role in endotheliotropism; putative CC chemokine motifs; associated with gH and gL	Akter *et al.* (2003); Hahn *et al.* (2004); Wang and Shenk (2005)
UL130		Virion glycoprotein with role in endotheliotropism; associated with gH and gL	Akter *et al.* (2003); Hahn *et al.* (2004); Patrone *et al.* (2005); Wang and Shenk (2005)
UL131A		Virion protein with role in endotheliotropism; associated with gH and gL	Akter *et al.* (2003); Hahn *et al.* (2004); Adler *et al.* (2006)
UL132		Putative membrane glycoprotein	
UL148		Putative membrane glycoprotein	
UL147A		Putative membrane protein	
UL147		UL146 family; putative secreted glycoprotein; putative CXC chemokine	Prichard *et al.* (2001)
UL146		UL146 family; secreted glycoprotein; CXC chemokine	Penfold *et al.* (1999); Prichard *et al.* (2001)
UL145			
UL144		Putative membrane glycoprotein; TNF receptor homologue	Benedict *et al.* (1999)
UL142		UL18 family; putative membrane glycoprotein; MHC-I homologue; inhibits NK cell cytotoxicity	Davison *et al.* (2003a); Wills *et al.* (2005)
UL141		UL14 family; membrane glycoprotein; inhibits NK cell cytotoxicity by downregulating CD155	Davison *et al.* (2003a); Tomasec *et al.* (2005)
UL140		Putative membrane protein	
UL139		Putative membrane glycoprotein	
UL138		Putative membrane protein	
UL136		Putative membrane protein	
UL135		Putative secreted protein	
UL133		Putative membrane protein	
UL148A		Putative membrane protein	
UL148B		Putative membrane protein	
UL148C		Putative membrane protein	
UL148D		Putative membrane protein	
UL150		Putative secreted protein	
IRS1		US22 family; immediate early transcriptional activator; tegument protein; involved in shutoff of host protein synthesis	Stasiak and Mocarski (1992); Romanowski and Shenk (1997); Romanowski *et al.* (1997); Child *et al.* (2004)
US1		US1 family; duplicated TT virus ORF2 motif	
US2		US2 family; membrane glycoprotein; causes selective degradation of MHC-I and MHC-II	Jones *et al.* (1995); Wiertz *et al.* (1996b); Jones and Sun (1997); Machold *et al.* (1997); Tomazin *et al.* (1999); Gewurz *et al.* (2001); Gewurz *et al.* (2002)

Table 14.1. (*cont.*)

HCMV[a]	HHV-6[a]	Function[b]	Selected references[c]
US3		US2 family; immediate early gene; membrane glycoprotein; inhibits processing and transport of MHC-I and MHC-II	Jones *et al.* (1996); Ahn *et al.* (1996); Hegde *et al.* (2002); Zhao and Biegalke (2003); Misaghi *et al.* (2004)
US6		US6 family; putative membrane glycoprotein; inhibits TAP-mediated peptide transport	Ahn *et al.* (1997); Lehner *et al.* (1997); Hewitt *et al.* (2001)
US7		US6 family; membrane glycoprotein	Huber *et al.* (2002)
US8		US6 family; membrane glycoprotein; binds to MHC-I	Huber *et al.* (2002); Tirabassi and Ploegh (2002)
US9		US6 family; membrane glycoprotein	Huber *et al.* (2002)
US10		US6 family; membrane glycoprotein; delays trafficking of MHC-I	Huber *et al.* (2002); Furman *et al.* (2002)
US11		US6 family; membrane glycoprotein; causes selective degradation of MHC-I	Jones *et al.* (1995); Wiertz *et al.* (1996a); Machold *et al.* (1997); Lilley and Ploegh (2004)
US12		US12 family; putative multiple transmembrane protein	
US13		US12 family; putative multiple transmembrane protein	
US14		US12 family; putative multiple transmembrane protein	
US15		US12 family; putative multiple transmembrane protein	
US16		US12 family; putative multiple transmembrane protein	
US17		US12 family; putative multiple transmembrane protein	
US18		US12 family; putative multiple transmembrane protein	Guo *et al.* (1993)
US19		US12 family; putative multiple transmembrane protein	Guo *et al.* (1993)
US20		US12 family; putative multiple transmembrane protein	Guo *et al.* (1993)
US21		US12 family; putative multiple transmembrane protein	
US22		US22 family; tegument protein; released from cells	Mocarski *et al.* (1988); Adair *et al.* (2002)
US23		US22 family	
US24		US22 family	
US26		US22 family	
US27		GPCR family; membrane protein	Fraile-Ramos *et al.* (2002)
US28		GPCR family; membrane protein; broad spectrum CC chemokine receptor; mediates cellular migration	Neote *et al.* (1993); Gao and Murphy (1994); Streblow *et al.* (1999)
US29		Putative membrane glycoprotein	
US30		Putative membrane glycoprotein	
US31		US1 family; duplicated TT virus ORF2 motif	
US32		US1 family; duplicated TT virus ORF2 motif	
US34		Putative secreted protein	
US34A		Putative membrane protein	
TRS1		US22 family; immediate early transcriptional activator; tegument protein; involved in shutoff of host protein synthesis and capsid assembly	Stasiak and Mocarski (1992); Romanowski and Shenk (1997; Romanowski *et al.* (1997); Child *et al.* (2004; Adamo *et al.* (2004)

[a] The layout here and in Fig. 14.1 is that derived by Davison *et al.* (2003a,b) and Dolan *et al.* (2004) for genes encoding functional proteins in wild type HCMV. A substantial number of additional protein-coding regions have been proposed (Chee *et al.*, 1990; Yu *et al.*, 2003; Murphy *et al.*, 2003a,b; Varnum *et al.*, 2004), and are not included because their status is considered generally less secure. The region between UL74 and UL75 may contain a small, rightward-oriented protein-coding exon (Scalzo *et al.*, 2004), and is also omitted. Homologous genes are in the same row. Blanks indicate the absence of detectable homologues. Bold font indicates HCMV genes that proved essential for growth in fibroblast cell lines in the study of Yu *et al.* (2003). Dunn *et al.* (2003b) also found that UL48, UL71, UL76, UL94 and UL96 are essential.

[b] Core genes (those inherited from the common ancestor of *Alpha-*, *Beta-* and *Gammaherpesvirinae*) are shaded, and functions take into account what is known about the homologue in the best characterized herpesvirus, which is often HSV-1 (see Chapter 2, Table 2.2). For genes conserved between HCMV and HHV-6, the information almost exclusively concerns the former. A blank indicates the absence of functional information.

[c] References primarily concern published data on gene identification and protein characterization, and are not exhaustive. Information on other aspects (e.g. dispensability, sequence variation or transcription) is generally not included. References to HHV-6 data (or, failing this, HHV-7 data) are denoted by square brackets.

[d] Positional homologue with similar functional characteristics.

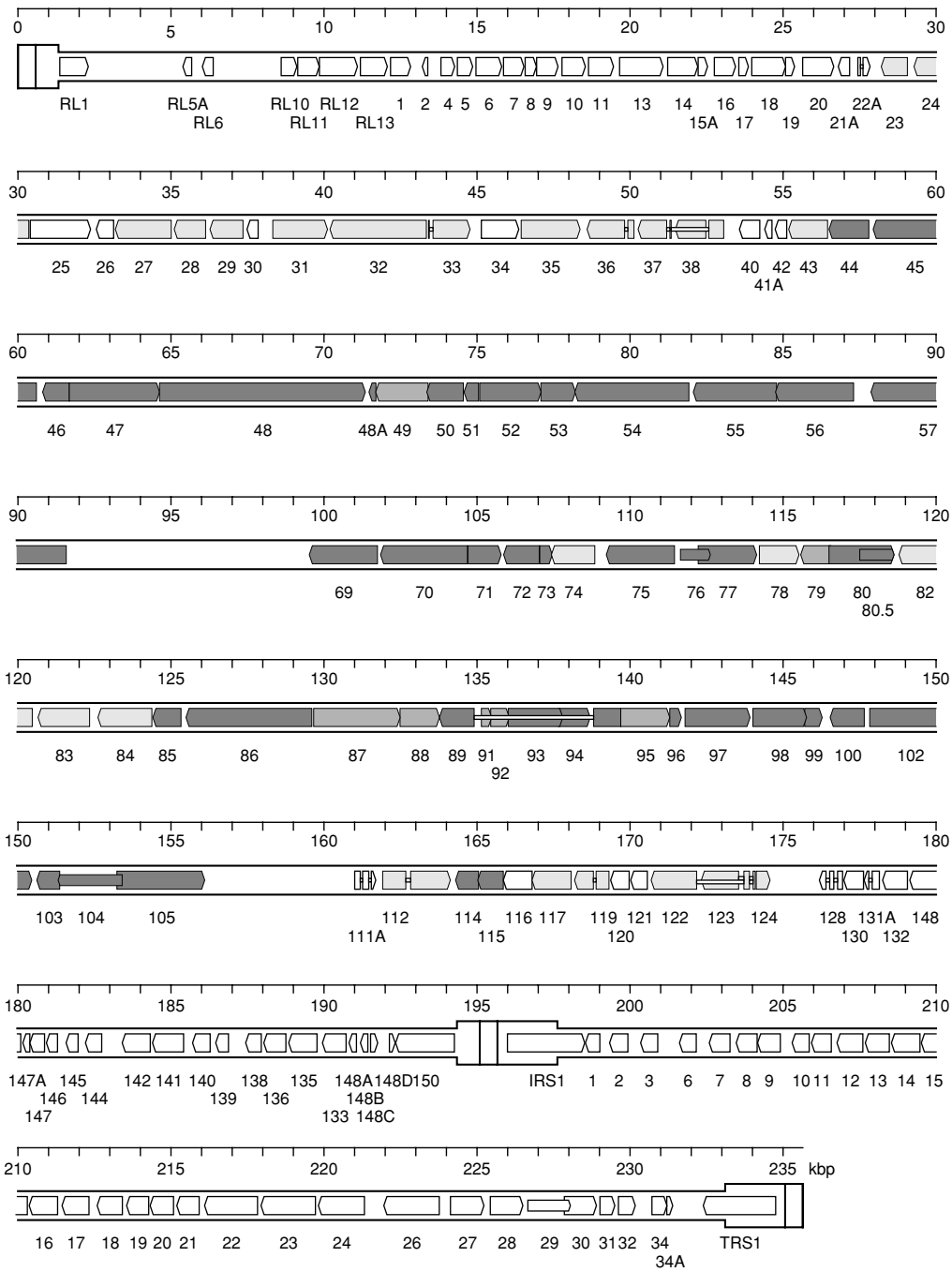

Fig. 14.1. Genetic content of wild-type HCMV, based on an interpretation of the genome sequence of strain Merlin (Dolan *et al.*, 2004), which in turn depended on analyses carried out by Chee *et al.* (1990), Cha *et al.* (1996) and Davison *et al.* (2003a, b). The inverted repeats TR_L/IR_L and TR_S/IR_S (which contain the *a* sequence depicted by a rectangle) are shown in a thicker format than U_L and U_S. Protein-coding regions are indicated by arrows, with gene nomenclature below. Introns are shown as narrow white bars. Genes corresponding to those in TR_L/IR_L and TR_S/IR_S of strain AD169 are given their full nomenclature, but the UL and US prefixes have been omitted from UL1–UL150 and US1–US34A. Core genes derived from the ancestor of *Alpha*, *Beta-* and *Gammaherpesvirinae* are shaded dark grey, additional genes derived from the ancestor of the *Beta-* and *Gammaherpesvirinae* are shaded mid grey, and other genes conserved between HCMV and HHV-6 are shaded light grey. White genes are unique to HCMV TRS1, US26 and US22 may be the counterparts of HHV-6 U95, DR1 and DR6, respectively, but are shown as unique.

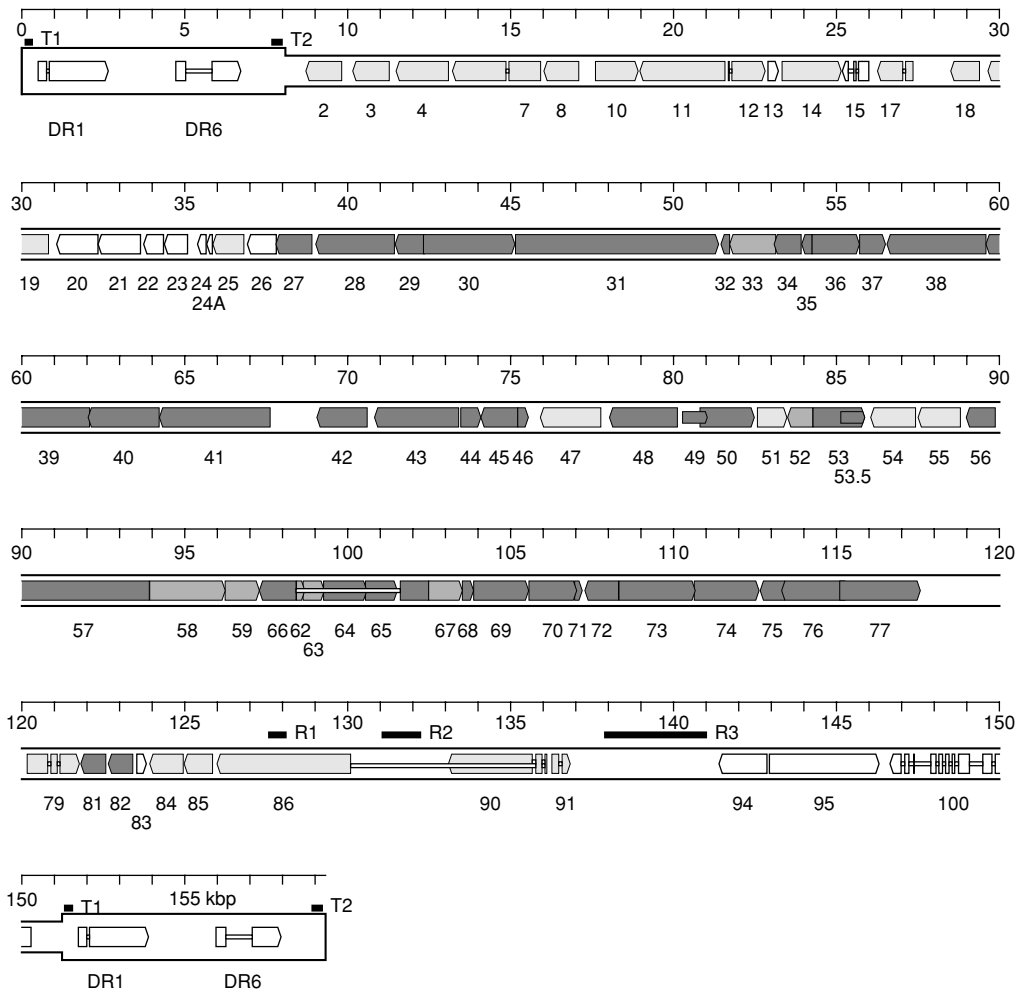

Fig. 14.2. Genetic content of HHV-6, based on strain U1102 of variant A (Gompels *et al.*, 1995) as modified by Megaw *et al.* (1998) and updated by the reintroduction of one gene (U83) and the incorporation of the splicing pattern for another (U86). The terminal direct repeat is shown in a thicker format than the unique region. Protein-coding regions are indicated by arrows, with gene nomenclature below. Introns are shown as narrow white bars. Genes in the direct repeat are given their full nomenclature, but the U prefix has been omitted from genes in the unique region. Core genes derived from the ancestor of *Alpha*, *Beta*- and *Gammaherpesvirinae* are shaded dark grey, additional genes derived from the ancestor of the *Beta*- and *Gammaherpesvirinae* are shaded light grey, and other genes conserved between HCMV and HHV-6 are shaded light grey. White genes are specific to HHV-6. U95, DR1 and DR6 may be the counterparts of HCMV TRS1, US26 and US22, respectively, but are shown as unique. Reiterated sequences T1, T2, R1, R2 and R3 are indicated by black bars.

The genes shared by HCMV and HHV-6 are collinear, probably as a result of being inherited from a common ancestor, but it is difficult to specify their number precisely. The best estimate, 73 genes, consists of 40 herpesvirus core genes, six genes inherited by the *Beta*- and *Gammaherpesvirinae*, and 27 genes specific to HCMV and HHV-6 (and HHV-7). The last class can be subdivided into 22 genes whose predicted amino acid sequences are detectably conserved (albeit weakly in some cases) in both genomes, along with the major immediate early gene (HCMV UL123) and the adjacent UL124 which lack amino acid similarity but correspond in position and orientation to HHV-6 genes (U90 and U91) with which they share properties, and three members of the US22 gene family (see below) that lack positional counterparts but have amino acid sequence counterparts elsewhere in the genome. HHV-6 U95, DR1 and

Fig. 14.3. Amino acid sequence alignments of proteins encoded by HCMV and HHV-6 genes putatively derived from a cellular OX-2 gene. (A) Conserved region in the ectodomains of human OX-2 (accession P41217), the HHV-6 and HHV-7 U85 proteins and cytomegalovirus UL119 proteins. The region includes residues 150–208 and 106–176 of the HCMV and HHV-6 proteins, respectively. Bold residues are conserved between OX-2 and one or both of the U85 proteins, or between both U85 proteins and at least two UL119 proteins. Three residues conserved throughout are indicated at the foot of the alignment. (B) Conserved residues in two regions of the primate cytomegalovirus UL120 and UL121 proteins. The entire sequence of each protein is shown. Bold residues are conserved in at least three orthologous proteins and at least one non-orthologous protein. Predicted signal and transmembrane sequences are indicated in grey and by underlining, respectively. The SCMV sequences are derived from Chang *et al.* (1995). Residues that are partially conserved between the OX-2-related proteins in (A) and the UL120 or UL121 proteins are indicated by colons.

DR6 may be the counterparts of HCMV TRS1, US26 and US22, respectively (Gompels *et al.*, 1995), but are not indicated as conserved in Figs. 14.1 and 14.2 or Table 14.1 because the precise correspondence is unclear. With these three exceptions, all of the genes inherited by HCMV and HHV-6 from their common ancestor are located between UL23 and UL124 in HCMV and U2 and U91 in HHV-6. Genes flanking this betaherpesvirus-common set are unique to each virus. Nonetheless, each virus possesses a number of genes within the conserved region that appear to be unique. These include genes lacking sequence and positional counterparts (e.g., HCMV UL116 and HHV-6 U83), genes whose positional counterparts lack sequence or functional similarities (e.g., HCMV UL34 and HHV-6 U13), and genes that are related to others and probably arose by duplication (e.g., HCMV UL25).

Gene duplication

Gene duplication has been employed widely by large eukaryotic DNA viruses and their hosts as a means of generating diversity (Prince and Pickett, 2002). Table 14.2 lists the 13 gene families that appear to have arisen in HCMV. Only four or five families have representatives, and each has fewer members, in HHV-6. The genes that have arisen by duplication appear to function in various specific aspects of the host response to infection. It is interesting to note evidence for duplication in the more recent evolution of these lineages, in the form of additional family members. For example, HHV-7 has two counterparts of HHV-6 U55 (termed U55A and U55B; Nicholas, 1996), CCMV lacks a counterpart to HCMV UL1 and has two additional counterparts of UL146 (Davison *et al.*, 2003a), and RhCMV and

Table 14.2. Gene families in HCMV and HHV-6

Family	HCMV	HHV-6	Features and putative functions
RL11	RL5A, RL6, RL11, RL12, RL13, UL1, UL4, UL5, UL6, UL7, UL8, UL9, UL10, UL11	None	Most are membrane glycoproteins; most share a putative immunoglobulin domain with certain human adenovirus E3 proteins
UL14	UL14, UL141	None	Membrane glycoproteins containing an immunoglobulin domain; UL141 is involved in NK cell evasion
UL18	UL18, UL142	None	MHC-I-related membrane glycoproteins; involved in immune evasion
US22	UL23, UL24, UL26, UL28, UL29, UL36, UL43, US22, US23, US24, US26, IRS1, TRS1	U2, U3, U7, U8, U17, U25, U95, DR1, DR6	Tegument proteins; involved in modulation of the cellular response
UL25	UL25, UL35	U14	Tegument proteins
GPCR	UL33, UL78, US27, US28	U12, U51	Chemokine receptors; some may have been captured independently
DURP	UL31, UL72, UL82, UL83, UL84	U10, U45, U54, U55 (U55A, U55B in HHV-7)	Derived from dUTPase; some are tegument proteins; multiple roles in modulating cellular responses
UL120	UL120, UL121, possibly UL119	Possibly U85	Membrane glycoproteins; possibly derived from OX-2
UL146	UL146, UL147	None	CXC chemokines
US1	US1, US31, US32	None	Related to TT virus ORF2
US2	US2, US3	None	Membrane glycoproteins; roles in immune evasion
US6	US6, US7, US8, US9, US10, US11	None	Membrane glycoproteins; roles in immune evasion
US12	US12, US13, US14, US15, US16, US17, US18, US19, US20, US21	None	Multiple transmembrane proteins

SCMV have additional members of the GPCR family corresponding to the US27 and US28 subset (Martin, 2000; Penfold *et al.*, 2003; Hansen *et al.*, 2003; Sahagun-Ruiz *et al.*, 2004). The potential for rapid evolution of gene family members is illustrated by the observation that certain examples (e.g., UL146 and some of the RL11 family) are among the most variable HCMV genes (Murphy *et al.*, 2003b; Dolan *et al.*, 2004).

It is likely that gene duplication in *Betaherpesvirinae* is more common than can be detected by amino acid sequence similarity, since it is possible for duplicated genes to diverge so extensively that similarity is obliterated. Consideration of two regions potentially in this category exemplifies the difficulties of defining evolutionary origins in such cases. In the first region, the three genes UL40-UL42 in HCMV are positionally equivalent to the six genes U20-U24A in HHV-6. Although the proteins encoded by these genes share no sequence similarity, the fact that all are predicted to be membrane-associated raises the possibility that the genes that encode them are derived from a single ancestor. It is interesting to note that one of the three HHV-6 genes (U22, U83 and U94) lacking counterparts in HHV-7 is located in this region. The analysis summarized in Fig. 14.3 sheds light on a second region, which contains the three genes UL119-UL121 in HCMV and the single gene U85 in HHV-6, all encoding putative membrane glycoproteins. The U85 protein is related to the cellular OX-2 surface glycoprotein, with greatest similarity apparent in an immunoglobulin domain (Gompels *et al.*, 1995). Atalay *et al.* (2002) also noted that an HCMV Fc-receptor encoded by HCMV UL119 contains an immunoglobulin domain. The alignment in Fig. 14.3(a) draws on data from several *Betaherpesvirinae* to support the view that U85 and UL119 are related, but with the latter diverging from OX-2 to a greater extent than the former. The alignment in Fig. 14.3(b) shows that the pair of genes adjacent to UL119 (UL120 and UL121) are distantly related to each other, and thus comprise a novel HCMV gene family. The colons between residues in Fig. 14.3(a) and Fig. 14.3(b) hint that UL120 and UL121 may be related to UL119 via the C-terminal portion of the immunoglobulin domain, although the degree of conservation is marginal. Thus, it appears that U85 and UL119 are both derived from a cellular OX-2 gene, and it is possible that UL120 and UL121 may also have arisen via duplication events involving the ancestor of UL119.

Gene capture

The simplest explanation for the presence in HCMV of genes that have counterparts in the cellular genome is that

of gene capture, a process which appears to involve insertion into the viral genome of a cDNA copy of a cellular mRNA or pre-mRNA, since most such genes lack introns. Recombination events of this sort probably occur commonly on an evolutionary scale, but few would result in viable virus, even fewer would provide a growth advantage, and even fewer still would become fixed in viral populations. The advantages of gene capture are illustrated in the case of cellular cyclo-oxygenase-2, which is involved in prostaglandin synthesis. Expression of this gene facilitates growth of HCMV (Speir *et al.*, 1998; Zhu *et al.*, 2002; Mocarski, 2002), and, unlike HCMV and CCMV, RhCMV has a copy in its genome (Hansen *et al.*, 2003). Gene capture has occurred throughout herpesvirus evolution, with ancient examples such as the dUTPase and uracil-DNA glycosylase genes common to the *Alpha-*, *Beta-* and *Gammaherpesvirinae*, more recent examples in certain of the GPCR family common to the *Betaherpesvirinae*, and even more recent examples such as the UL146 chemokine gene in primate cytomegaloviruses. Some, perhaps all, of the gene families have arisen from captured genes.

In addition to incorporating cellular genes, *Betaherpesvirinae* appear to have exchanged genetic information with other viruses. Thus, HHV-6 U94 is related to the Rep gene of *Parvoviridae*, but lacks a counterpart in HHV-7 (Thomson *et al.*, 1991). The RL11 family proteins of primate cytomegaloviruses share a putative immunoglobulin domain with a glycoprotein family encoded in the E3 region of primate *Adenoviridae* (Davison *et al.*, 2003b). A previously unreported finding concerns the primate cytomegalovirus US1 gene family, whose functions are unknown. Hijikata *et al.* (1999) noted a motif (WX_7 HX_3 $CXCX_5$ H) that is conserved near the N terminus of VP2 in a small, single-stranded DNA virus, chicken anaemia virus (CAV; *Circoviridae*). The N-terminal region of the ORF2 protein in the related TT viruses (TTVs) and TT-like viruses, which are common in humans, also contain this motif. Figure 14.4 shows that the N-terminal regions of proteins specified by the primate cytomegalovirus US1 gene family contain two copies of this motif, each of which, as suggested from the nature of the conserved residues, might coordinate a metal ion. Parenthetically, it is interesting to note that the motif is located within an extended region identified by Peters *et al.* (2002) as conserved between CAV VP2 and cellular protein-tyrosine phosphatases (PTPases). However, the significance of the relationship to PTPases is dubious, since most of the PTPase residues conserved in CAV VP2 are not conserved in the TTV ORF2 protein, and proposed catalytic residues are significantly diverged in VP2 and essentially absent from the ORF2 protein. In any case, the known PTPase catalytic residues are C-terminal to the region aligned with the proposed catalytic residues in VP2, and well outside the conserved region. It is surprising, therefore, that Peters *et al.* (2002) were able to show that VP2 and the ORF2 protein of a TT-like virus have PTPase activity.

Variation

HCMV isolates exhibit a high degree of variation in a wide range of genes (Murphy *et al.*, 2003b; Dolan *et al.*, 2004; Pignatelli *et al.*, 2004). The most variable genes potentially encode proteins that are secreted or associated with membranes, and therefore most exposed to selection by the immune system. Attempts to associate certain alleles with particular pathogenic properties have been largely unsuccessful. This may reflect the multifactorial nature of such properties, and the fact that recombination has played a part in HCMV evolution (Arav-Boger *et al.*, 2002; Rasmussen *et al.*, 2003). Limited studies of variation have also been carried out in HHV-6 (Dominguez *et al.*, 1996; Stanton *et al.*, 2003) and HHV-7 (Chan *et al.*, 2003).

Gene function

Most effort on gene function has been carried out on HCMV, but advances with other cytomegaloviruses have been substantial. Indeed, the use of the powerful bacterial articificial chromosome technology for generating mutants was innovated for MCMV (Messerle *et al.*, 1997) before it was applied to other cytomegaloviruses, including HCMV (Borst *et al.*, 1999; McGregor and Schleiss, 2001; Yu *et al.*, 2002; Chang and Barry, 2003; Murphy *et al.*, 2003b). Studies on non-human cytomegaloviruses form a vital basis for assessing the functions of genes in pathogenesis by means that cannot be applied for HCMV. The parallels are incomplete, however, as even the closest relative of HCMV that could be used practically as an animal model, RhCMV, exhibits genetic differences in comparison with HCMV. With regard to the effects of infection with *Betaherpesvirinae* on cellular transcription, several microarray analyses have been carried out (Zhu *et al.*, 1998; Kenzelmann and Muhlemann, 2000; Simmen *et al.*, 2001; Browne *et al.*, 2001). Further details on HCMV gene function are given in Chapter 15.

Virion structure

In all *Herpesviridae,* the genome is packaged within the icosahedral capsid, which is embedded in a dense proteinaceous matrix termed the tegument, and in turn surrounded by a lipid envelope extensively decorated with viral

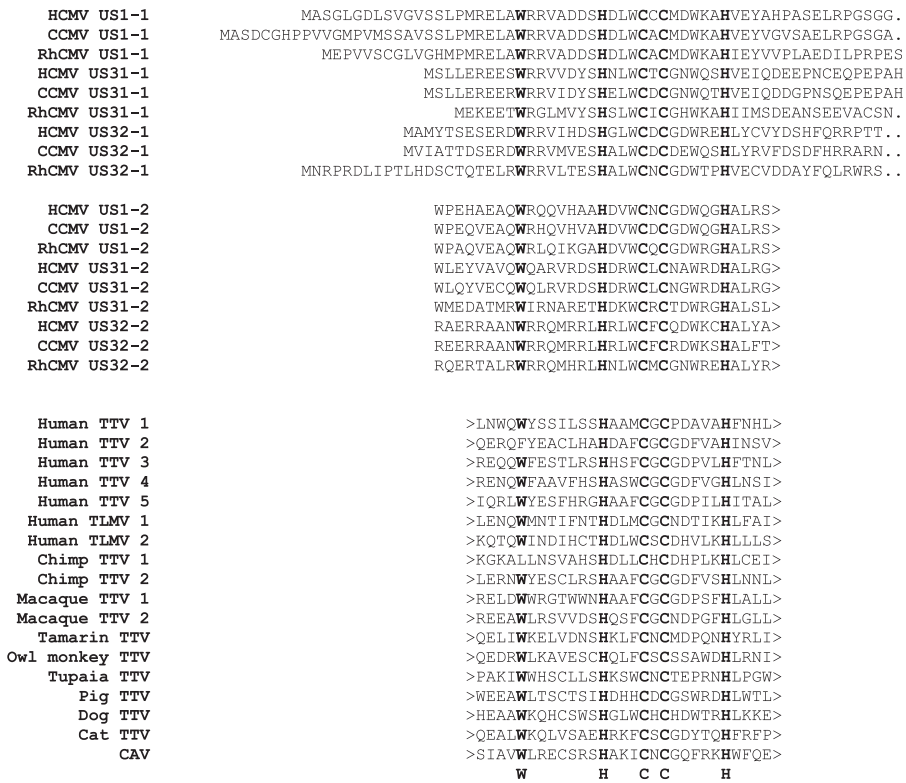

Fig. 14.4. Amino acid sequence alignments of the $WX_7HX_3CXCX_5H$ motif near the N termini of primate cytomegalovirus proteins encoded by the US1 gene family (US1, US31 and US32), the ORF2 proteins of TT viruses (TTV) of various hosts (five, two and two types for humans, chimpanzees and macaques, respectively) and TTV-like miniviruses (TLMV) of humans (two types), and VP2 of chicken anaemia virus (CAV). The cytomegalovirus sequences in the upper two panels commence at the N-terminus and proceed continuously for each virus, so that the two copies of the motif are aligned sequentially. The TTV, TLMV and CAV proteins contain a single copy of the motif. Sequences were derived via the information given in Fig. 2 in Okamoto *et al.* (2002). Arrowheads indicate that the sequences extend further towards the C terminus (cytomegalovirus proteins) or both termini (TTV, TLMV and CAV proteins). The motif is shown at the foot of the alignment.

glycoproteins (see Chapter 3). Virion morphogenesis commences in the host cell nucleus, beginning with assembly of the spherical procapsid by condensation of the capsid and scaffold proteins (see Chapter 20). By analogy with HSV-1 (Newcomb *et al.*, 1996), proteolytic maturation of the HCMV procapsid separates the scaffold from the inside surface of the capsid, giving rise to a more angular particle, while packaging of the viral genome leads to egress of the scaffold component and formation of mature C-capsids (Irmiere and Gibson, 1985). A-capsids and B-capsids are also found in great abundance in the nucleus and in mature virions (Irmiere and Gibson, 1983). A-capsids are devoid of scaffold or DNA, and are thought to be the product of aborted packaging. B-capsids are mature particles containing large or small scaffold cores that are thought to be either precursors of DNA packaging or byproducts of failed packaging (Fig. 14.5(a)). Mature capsids (A, B and C) bud out of the nucleus, and are thought to undergo a process of envelopment and de-envelopment as they traverse the nuclear membrane (see Chapter 20). The maturing virion then acquires its tegument in perinuclear compartments termed tegusomes. Finally, capsids bud into Golgi-derived cytoplasmic compartments, gaining their lipid membrane and envelope glycoproteins in the process, and exit the cell by the exocytotic pathway.

The genome

HCMV has a larger genome than members of the *Alpha-* and *Gammaherpesvirinae*, and it is consequently more tightly packaged within the capsid. However, this is not a generalized feature of the *Betaherpesvirinae,* as the genomes of HHV-6 and HHV-7 are smaller. Nonetheless, the requirement for orderly ingress and egress of the genome, through

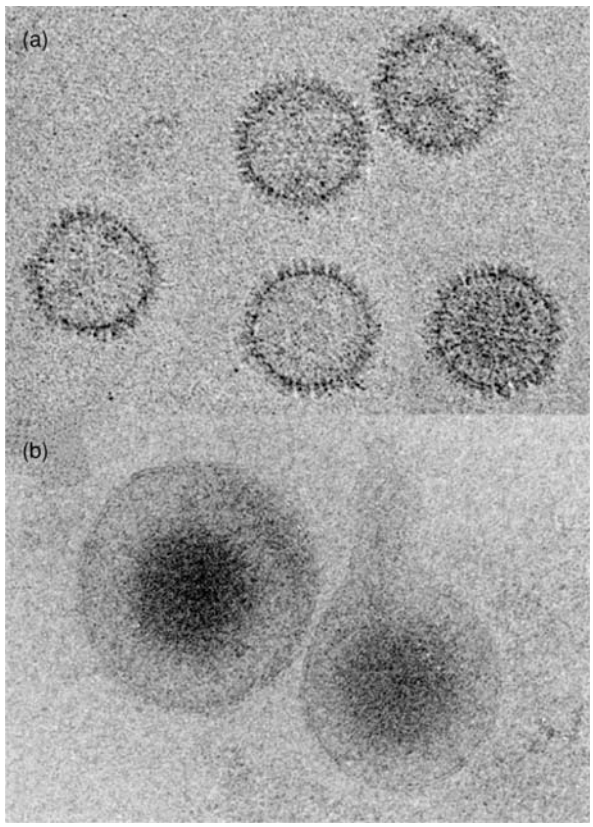

Fig. 14.5. Cryo-micrographs of (a) purified HCMV B-capsids and (b) purified HCMV virions showing the characteristic swirling pattern of the packaged genomic DNA (from Bhella *et al.*, 2000; with permission from Academic Press).

a portal complex, suggests a high degree of organization in genome packaging.

Imaging of HCMV virions by cryo-microscopy allows the packaged DNA molecule to be visualized (Fig. 14.5(b)), revealing the characteristic swirled, striated and punctate array patterns seen in many *Herpesviridae*, as well as in the double-stranded DNA bacteriophages T4, T7, λ and p22 (Bhella *et al.*, 2000). These patterns suggest a mode of packaging in which DNA enters the capsid and spools around the inner surface, winding inwards, towards the centre, in successive layers (Booy *et al.*, 1991; Zhou *et al.*, 1999). Measurements indicate that the HCMV genome is packaged in layers approximately 23 Å apart. Assuming hexagonal packing, which is the preferred conformation of DNA fibres at this packing density, this corresponds to an average interhelix spacing of 26 Å. HSV-1 virions show an interlayer spacing of 26 Å, corresponding to an interhelix distance of 30 Å. At such packing densities, the DNA would be expected to be in a liquid-crystalline state, with possible

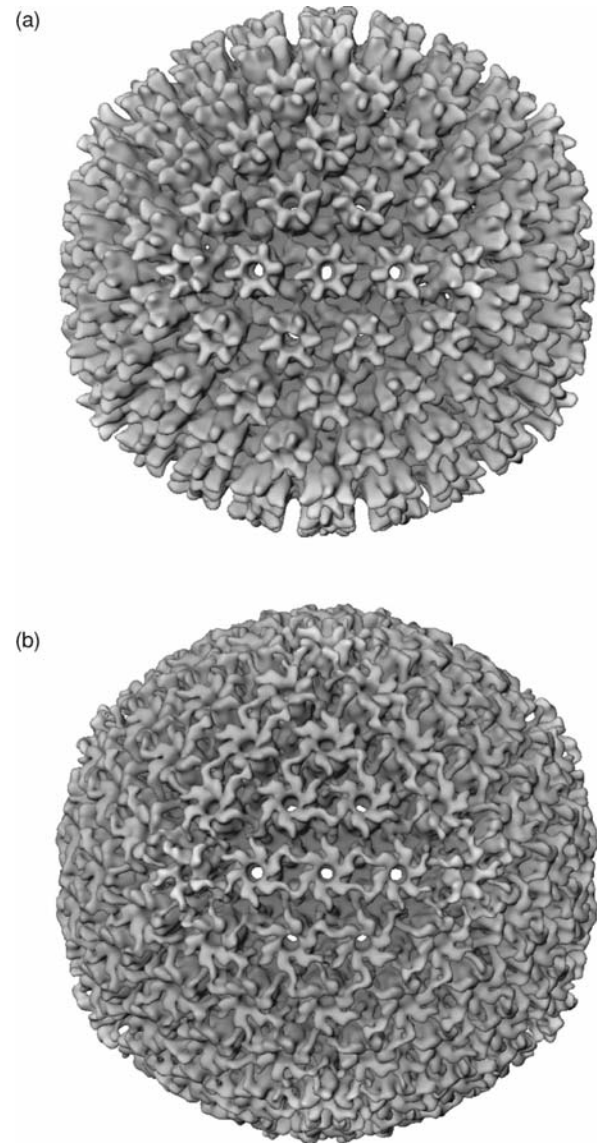

Fig. 14.6. Three-dimensional reconstructions of (a) the HCMV B-capsid (adapted from Butcher *et al.*, 1998; with permission from Academic Press), and (b) the HCMV virion (adapted from Zhou *et al.*, 1999; with permission from the American Society for Microbiology). Both structures are viewed along the icosahedral twofold symmetry axes and are radially depth-cued so that darker regions are closer to the center of the particle and lighter regions are further away. (See color plate section.)

local transitions to three-dimensional hexagonal packing in those viruses with a tighter packing density. However, curvature of the packaged DNA would prevent extensive transition to a crystalline state, suggesting that 26 Å is the upper limit of packing density achievable. In order to

package its genome, HCMV has evolved to produce a larger capsid, with a diameter of 130 nm rather than 125 nm (Butcher *et al.*, 1998).

The capsid

Investigation of B-capsid structure in HCMV strain AD169 by cryo-microscopy and image reconstruction (Fig. 14.5(a)) has revealed an architecture very similar to that of HSV-1, consisting of a hexamer-pentamer clustered T=16 icosadeltahedral shell (Butcher *et al.*, 1998). The capsid is complex, consisting of four structural proteins: the major capsid protein (MCP; encoded by UL86), the minor capsid protein (mCP; UL85), the minor capsid protein-binding protein (mC-BP; UL46) and the smallest capsid protein (SCP; UL48A) (Gibson, 1983; Irmiere and Gibson, 1985; Sedarati and Rosenthal, 1988; Gibson, 1996; Gibson *et al.*, 1996a; Gibson *et al.*, 1996b). In addition, the portal complex, which is presumed to occupy one of the fivefold vertices, is considered a capsid component.

The major structural components of the capsid are the capsomeres, termed pentons and hexons. These large, turret-like structures are composed primarily of five or six copies of the MCP, respectively. In total, there are 150 hexons occupying positions of local six-fold symmetry, and 12 pentons, located at the icosahedral five-fold symmetry axes (Fig. 14.6(a)). The hexons and pentons are 15 nm tall and have a central channel running through them that is constricted some 7.4 nm from the capsid floor, which is also composed of the MCP and appears thicker in HCMV than in HSV-1. The tips of each hexon are decorated with six copies of SCP (Yu *et al.*, 2005), a feature also found in HSV-1, in which the hexons are capped by the homologous protein, VP26 (Booy *et al.*, 1994; Trus *et al.*, 1995; Zhou *et al.*, 1995). A small capsid protein is also associated with the tips of hexons in HHV-8, but is also present at the tips of pentons (Trus *et al.*, 2001). Between the HCMV capsomeres, lying on the capsid floor at positions of local threefold symmetry, are the triplexes. These heterotrimeric structures are composed of two copies of the mCP and one copy of the mC-BP. The triplexes are critical for capsid morphogenesis, most likely linking together the capsomeres in the procapsid, directing assembly, and stabilizing the structure prior to maturation.

The tegument

Perhaps the most striking morphological difference between HCMV and HSV-1 is the extent of icosahedrally ordered tegument in the virion. Cryomicroscopy and image reconstruction have shown that in HSV-1 the only additional, icosahedrally ordered protein that is present in the virion but not the B-capsid is located at the fivefold vertices, where a single rod-like structure connects the tips of each penton MCP (VP5) subunit with the adjacent triplex and its nearest neighboring triplex (Zhou *et al.*, 1999). However, HCMV has an extensive network of icosahedral tegument that, while appearing similar to that found at the HSV-1 penton, also decorates the hexons (Fig. 14.6(b)) (Chen *et al.*, 1999). Appearing as a bridge of density, approximately 120 Å in length and 20–30 Å in diameter, the icosahedrally ordered tegument is anchored to the tip of the hexons and pentons and the top of the adjacent triplex. Investigation of the cytoplasmic B-capsids of SCMV revealed a similar pattern of tegumentation (Trus *et al.*, 1999), although independent reconstruction of lightly, moderately and heavily tegumented capsids suggests that this density is composed of at least two protein species. The first is a capsomere capping protein, which is found at the tips of both the hexons and pentons and therefore binds to the MCP rather than the SCP, which is not present on the penton. The second protein is attached to the capsomere capping protein and the adjacent triplex. The rest of the tegument appears to not be rigidly icosahedral in distribution, but this does not preclude the existence of further order.

Many tegument proteins play roles in the early stages of virus infection and are therefore packaged within the virion to ensure their presence upon infection of a new host, and many are phosphorylated (Roby and Gibson, 1986). The most abundant proteins are the basic phosphoprotein (pp150; encoded by UL32), which is O-glycosylated (Benko *et al.*, 1988) and binds directly to the capsid through its N-terminal one-third (Baxter and Gibson, 2001), the lower matrix protein (pp65; UL83), the upper matrix protein (pp71; UL82), the membrane-associated myristylated protein (pp28; UL99; Sanchez *et al.*, 2000), and the high molecular weight tegument protein (UL48) and its binding protein (UL47). The requirement to incorporate specific proteins suggests that tegument morphogenesis may involve specific interactions between individual components, leading to the formation of a structure that is at least partially ordered. This hypothesis is borne out by the finding that HCMV infections in cell culture give rise to production of many particles composed of enveloped tegument lacking a capsid (dense bodies), suggesting that the tegument can spontaneously self-assemble, possibly around aggregates of pp65 (Irmiere and Gibson, 1983). Moreover, immuno-gold labelling experiments on thin sections of virus-infected cells can reproducibly locate tegument proteins to the outer or inner regions of the tegument (Landini *et al.*, 1987). Logically, specific proteins might be expected to be located at the outer edge of the tegument, since in most enveloped viruses there is a requirement for a specific

interaction between viral matrix proteins and the cytoplasmic tail of envelope glycoproteins. Indeed, pp28 has been shown to be essential for envelopment, and may well serve this function (Silva *et al.*, 2003).

Envelope glycoproteins

Many HCMV genes encode putative membrane glycoproteins. However, few thus far have been identified as components of the virion. The most abundant protein species in the viral envelope have homologues throughout the *Herpesviridae*. These are distributed among three complexes: gCI, gCII and gCIII (Gretch *et al.*, 1988). gCI is composed of homodimers of glycoprotein B (gB; UL55), a type I integral membrane protein (Cranage *et al.*, 1986). gB is cleaved by furin into two fragments (gp55 and gp116), which remain covalently associated (Vey *et al.*, 1995). Dimerization of gB is disulfide bond dependent, and mass spectrometric analysis of proteolytic fragments of recombinant gB has revealed extensive disulfide bond formation, both between gB monomers and also between gp55 and gp116 (Eickmann *et al.*, 1998; Lopper and Compton, 2002). Along with gCII, gCI mediates cell attachment, a two-step process involving initial attachment to heparan sulfate, followed by a stabilizing interaction with another receptor (Boyle and Compton, 1998; see Chapter 16). gCII comprises two proteins: gM (UL100), a type III membrane protein, and gN (UL73), a type I membrane protein (Kari *et al.*, 1994; Mach *et al.*, 2000). gCII components are also heavily disulfide linked, although the presence of monomeric forms of these glycoproteins in the virion suggests that these bonds may not be essential for formation and function of the gCII complex (Kari *et al.*, 1990). gCIII includes a heterotrimer of gH (UL75) as well as a heterotrimer of these proteins plus a third component, gO (UL74), which lacks counterparts in *Alpha-* and *Gammaherpesvirinae* (Kaye *et al.*, 1992b; Spaete *et al.*, 1993; Huber and Compton, 1998; Li *et al.*, 1997). gCIII is involved in mediating fusion of viral and host cell membranes, in concert with gCI. In HHV-6, the third component of gCIII is gQ (U100) rather than gO (Mori *et al.*, 2003a).

In addition to glycoproteins that have counterparts throughout the *Herpesviridae*, HCMV-specific glycoproteins encoded by UL4 and RL10 have been identified as virion components (Chang *et al.*, 1989; Spaderna *et al.*, 2002). Analysis of the trafficking in cells of the RL10 protein in isolation from other HCMV proteins indicates that it may be necessary for this glycoprotein to form complexes with other membrane glycoproteins in order to facilitate incorporation into nascent virions. Similarly to the glycoproteins common to the *Herpesviridae*, the RL10 protein forms high molecular weight complexes in mature virions, possibly by disulfide bond formation.

Additional virion components

In addition to the genome, two small RNA molecules are present in the core of the virion, hybridized to the origin of DNA replication (Prichard *et al.*, 1998). These molecules may play a role in initiating DNA replication by providing a point of action for an RNase H-like enzyme, and digestion of the DNA:RNA hybrid could lead to formation of a primer in the form of the opened DNA or possibly a small fragment of the RNA. A number of mRNAs are also associated with virions, most likely located in the tegument (Bresnahan and Shenk, 2000a). The RNAs detected are specified by UL22A (also termed UL21.5) and RL13, and putative non-protein-coding regions encompassing ORFs UL106-UL109, RL2-RL5 and RL7 as defined by Chee *et al.* (1990). Translation of certain of these RNAs could ensure targeting of encoded proteins to the endoplasmic reticulum and Golgi network, before the viral genome becomes transcriptionally active. Alternatively, these molecules may be modulators of cellular immunity or structural components of the tegument. However, the biological significance of virion RNAs is questionable, since it is now known that incorporation of viral and cellular mRNAs occurs non-specifically, with the abundance of RNAs associated with the virion reflecting that in infected cells (Greijer *et al.*, 2000; Terhune *et al.*, 2004).

As there are no histone-like proteins present in herpesvirions to neutralize the charge of the packaged DNA molecule, some other mechanism must be employed. Detection of the polyamines spermine and spermidine in HCMV virions, and the finding that inhibition of polyamine biosynthesis inhibits virus growth at the level of virion assembly, has led to the suggestion that these molecules may fulfil this role (Gibson *et al.*, 1984). Other virion components contributed by the host cell include annexin II (Wright *et al.*, 1995), phospholipase A2 (Allal *et al.*, 2004), the complement control proteins CD55 and CD59 (Spear *et al.*, 1995) and β-2-microglobulin (McKeating *et al.*, 1987), which are associated with the virion envelope. β-2-microglobulin has also been detected in the tegument (Stannard, 1989), as has an actin-like protein (Baldick and Shenk, 1996). Mass spectrometric methods have recently enabled the proteins present in HCMV virions to be catalogued, including a number of viral proteins not detected hitherto and an impressive number of cellular proteins (Varnum *et al.*, 2004). However, caution needs to be exercised in assessing the biological relevance of proteins detected in low abundance by sensitive immunological or physical methods, since proving the absence of contamination with non-virion material is problematic.

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15

Betaherpes viral genes and their functions

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Introduction

Despite biological divergence, human cytomegalovirus (HCMV, HHV-5), on the one hand, and the three human roseolaviruses (HHV-6A, HHV-6B, HHV-7), on the other hand, share approximately 70 evolutionarily conserved and collinear genes (italics, Table 15.1). Mammalian betaherpesviruses probably have a common ancestor dating back over 50 million years. The genomes of all betaherpesviruses vary in the regions flanking a large conserved block of genes spanning UL23 to UL124 in HCMV. There appear to be two distinct evolutionary lineages represented by cytomegaloviruses and roseolaviruses infecting primates (Chapter 14). These lineages are not preserved in lower mammals such as rodents where divergence within the cytomegaloviruses is striking. Similarity between rodent cytomegaloviruses and either primate cytomegaloviruses or roseolaviruses is about the same, and includes the same set of 70 conserved genes. Thus, despite the fact that this subgroup of herpesviruses is the most highly distributed amongst mammals, evolutionary divergence is dramatic. The betaherpesvirus-common genes are composed of 41 herpesvirus core functions discussed in Chapters 3, 4 and 14 plus approximately 30 betaherpesvirus-specific gene products that are involved in replication and cell tropism. Biological similarities include tropism for hematopoietic cells in the myeloid lineage (Kondo *et al.*, 2002a; Sissons *et al.*, 2002). In addition to the 70 conserved replication genes human betaherpesviruses HCMV and HHV-6B also have latent genes with a common structure and genomic location (Kondo *et al.*, 1996, 2002b 2003b) suggesting evolutionary conservation in this important process as well. Although little is known about their functions, homologues of several betaherpesvirus-specific genes (UL49, UL79, UL87, UL88, UL91, UL92, and possibly UL95) are common to gammaherpesviruses (see Table 14.1). Thus these two groups are more closely related to each other than to any alphaherpesviruses. Outside of the betaherpesvirus-common genes, there is considerable diversity in this virus group, including the presence of an alphaherpesvirus-like DNA replication origin and origin binding protein in roseolaviruses as well as genes that have been retained only in certain lineages, such as the presence of an adeno-associated virus *rep* gene homologue in closely related HHV-6A and HHV-6B, but not HHV-7, a gene that is conserved as well in the distant rodent betaherpesvirus, rat cytomegalovirus. The presence of specific immunomodulatory genes in only a subset of cytomegaloviruses is also striking (Mocarski, 2002, 2004). How such an array of distinct gene products entered the betaherpesvirus lineage and why they have been retained remain unresolved questions.

As discussed in Chapters 14, the betaherpesvirus-conserved genes include the herpesvirus core set. These genes are clustered towards the center of viral genomes within the unique long (U_L) genomic component of HCMV (Table 15.1). The role of HCMV genes in replication has been systematically studied in variants (*var*) of two laboratory strains Towne*var*ATCC (Dunn *et al.*, 2003b) or AD169*var*ATCC (Yu *et al.*, 2003), and five categories of growth relative to wild-type (parental) virus, have been described: (1) replication better than wild-type (temperance), (2) replication the same as wild-type, (3) complete replication deficiency, (4) severely reduced replication efficiency, (5) slightly reduced replication efficiency (Table 15.1). The host cell type dictates the category for some genes (Dunn *et al.*, 2003b; Hahn *et al.*, 2004) and varying behavior has been observed when viral mutants are studied in fibroblasts, endothelial cells and retinal pigment epithelial cells. In addition to varying behavior in different cell types, HCMV exhibits sensitivity to the cell cycle state at the time of infection (Fortunato *et al.*, 2002) and some

Table 15.1. Summary information on human betaherpesvirus gene products

HCMV[a]	HHV-6[a]	HHV-7[a]	HCMV gene family or function[b]	Selected references[c]
RL1				
RL5A			RL11 family	
RL6			RL11 family	
RL10			Virion envelope glycoprotein	(Spaderna *et al.*, 2002; Spaderna *et al.*, 2004)
RL11			RL11 family; IgG Fc-binding glycoprotein	(Atalay *et al.*, 2002; Lilley *et al.*, 2001)
RL12			RL11 family; putative membrane glycoprotein	
RL13			RL11 family; putative membrane glycoprotein	(Yu *et al.*, 2002)
UL1			RL11 family	
UL2			Putative membrane protein	
UL4			RL11 family; translationally regulated virion glycoprotein	(Alderete *et al.*, 2001; Janzen *et al.*, 2002)
UL5			RL11 family; virion membrane protein?	(Varnum *et al.*, 2004)
UL6			RL11 family; putative membrane glycoprotein	
UL7			RL11 family; putative membrane glycoprotein	
UL8			RL11 family; putative membrane glycoprotein	
UL9*			RL11 family; putative membrane glycoprotein; temperance for fibroblasts	
UL10*			RL11 family; putative membrane glycoprotein temperance for RPE	
UL11			RL11 family; membrane glycoprotein	(Hitomi *et al.*, 1997)
UL13			Putative secreted protein	
UL14			UL14 family; putative membrane glycoprotein	
UL15A			Putative membrane protein	
UL16*			Membrane glycoprotein; inhibits NK cell cytotoxicity; MICA-ULBP ligand; temperance for RPE	(Dunn *et al.*, 2003a; Vales-Gomez *et al.*, 2003)
UL17				
UL18			UL18 family; putative membrane glycoprotein; MHC-I homologue; LIR-1 ligand	(Saverino *et al.*, 2004; Vales-Gomez *et al.*, 2005; Willcox *et al.*, 2003))
UL19				
UL20			T cell receptor γ chain homologue	
UL21A*¥			CC chemokine binding protein (also called UL20A and UL21.5); temperance for fibroblasts	(Wang *et al.*, 2004a)
UL22A¥			Virion protein, secreted glycoprotein (also called UL22.5)	(Varnum *et al.*, 2004)
*UL23**	U2	U2	US22 family; tegument protein; temperance for fibroblasts	(Adair *et al.*, 2002)
UL24	U3	U3	US22 family; tegument protein, necessary in HMVECs.	(Adair *et al.*, 2002) [(Kondo *et al.*, 2003a)]
UL25			UL25 family; tegument phosphoprotein	(Battista *et al.*, 1999; Zini *et al.*, 1999; Zini *et al.*, 2000)
UL26¥			US22 family; tegument protein; transcriptional activator of major immediate early promoter	(Stamminger *et al.*, 2002)
UL27	U4	U4	Maribavir resistance	(Chou *et al.*, 2004; Komazin *et al.*, 2003) [(Kondo *et al.*, 2003a)]
UL28¥	U7 × 2U7 × 1	U5 U7	US22 family	[(Kondo *et al.*, 2003a)]
UL29¶	U8	U8	US22 family, growth efficiency in RPE cells	
UL30¥	U9[d]			
UL31¶	U10	U10	[HHV-7 IE gene]	[(Menegazzi *et al.*, 1999)]

(*cont.*)

Table 15.1. (*cont.*)

HCMV[a]	HHV-6[a]	HHV-7[a]	HCMV gene family or function[b]	Selected references[c]
UL32†	U11	U11	Major tegument phosphoprotein (pp150); highly immunogenic; binds to capsids [HHV-6 p100]	(Baxter and Gibson, 2001; Sampaio *et al.*, 2005) [(Neipel *et al.*, 1992; Stefan *et al.*, 1997)]
UL33 × 1	U12 × 1	U12 × 1	GPCR-7TM family; constitutive signaling, envelope protein [HHV-6A, HHV-6B, HHV-7 are chemokine receptors]	(Casarosa *et al.*, 2003; Fraile-Ramos *et al.*, 2002); [(Isegawa *et al.*, 1998; Milne *et al.*, 2000; Nakano *et al.*, 2003)]
UL33 × 2	U12 × 2	U12 × 2		
UL34†	U13[d]	U13[d]	Represses US3 transcription	(Biegalke *et al.*, 2004; LaPierre and Biegalke, 2001)
UL35¶	U14	U14	UL25 family; tegument phosphoprotein; interacts with UL82 protein [HHV-7 IE gene]	(Liu and Biegalke, 2002; Schierling *et al.*, 2005; Schierling *et al.*, 2004) [(Menegazzi *et al.*, 1999; Stefan *et al.*, 1997)]
	U15	U15		
UL36 × 1	U17 × 1	U17 × 1	US22 family; immediate early protein, tegument protein; inhibitor of caspase-8-induced apoptosis (vICA). [HHV-6 IE gene]	(McCormick *et al.*, 2003; Patterson and Shenk, 1999; Skaletskaya *et al.*, 2001) [(Flebbe-Rehwaldt *et al.*, 2000; Mirandola *et al.*, 1998)]
UL36 × 2	U17 × 2	U17 × 2		
UL37 × 1¶			mitochondrial inhibitor of apoptosis (vMIA) protein.	(Goldmacher *et al.*, 1999; Hayajneh *et al.*, 2001a; McCormick *et al.*, 2003; Reboredo *et al.*, 2004; McCormick *et al.* 2005)
UL37 × 3	U18	U18	Immediate early glycoprotein. [HHV-6. HHV-7 IE gene]	(Adair *et al.*, 2003; Hayajneh *et al.*, 2001b) [(Menegazzi *et al.*, 1999; Mirandola *et al.*, 1998)]
UL38¶	U19	U19	virion glycoprotein	(Varnum *et al.*, 2004)[(Flebbe-Rehwaldt *et al.*, 2000; Menegazzi *et al.*, 1999; Mirandola *et al.*, 1998)]
UL40			Membrane glycoprotein; signal peptide binds HLA-E to inhibit NK cell cytotoxicity	(Tomasec *et al.*, 2000; Ulbrecht *et al.*, 2000; Wang *et al.*, 2002)
	U20	U20	Putative membrane glycoprotein	[(Mirandola *et al.*, 1998)]
	U21	U21	Putative membrane glycoprotein [directs MHC class I to lysosomes]	[(Hudson *et al.*, 2001)]
	U22	U22	Putative membrane glycoprotein	
	U23	U23	Putative membrane glycoprotein	
	U24	U24	Putative membrane protein	
	U24A	U24A	Putative membrane protein	
UL41A			Virion membrane protein (also called UL41.5)	(Varnum *et al.*, 2004)
UL42			Putative membrane protein	(Dargan *et al.*, 1997; Mocarski *et al.*, 1997)
UL43	U25	U25	US22 family; tegument protein	(Adair *et al.*, 2002)
	U26	U26	Putative multiple transmembrane protein	
UL44†	U27	U27	(core) DNA polymerase processivity subunit (PPS)	(Appleton *et al.*, 2004; Loregian *et al.*, 2004a; Loregian *et al.*, 2004b) [(Agulnick *et al.*, 1993; Lin and Ricciardi, 1998)]
UL45	U28	U28	(core) Tegument; Large subunit of ribonucleotide reductase homologue (enzymatically inactive); virion protein (RR1)	(Hahn *et al.*, 2002; Patrone *et al.*, 2003) [(Sun and Conner, 1999)]
UL46†	U29	U29	(core) Component of capsid triplexes (minor capsid binding protein; TRI1)	(Gibson *et al.*, 1996)
UL47¶	U30	U30	(core) Tegument; intracellular capsid transport; binds to UL48 protein? (LTPbp)	(Bechtel and Shenk, 2002)
UL48†	U31	U31	(core) Largest tegument protein; binds to UL47 protein?; intracellular capsid transport? (LTP) [HHV-7 IE gene]	(Ogawa-Goto *et al.*, 2002) [(Menegazzi *et al.*, 1999)]
UL48A†	U32	U32	(core) Located on tips of hexons in capsids; capsid transport? (SCP) (also called UL48.5)	(Lai and Britt, 2003; Yu *et al.*, 2005)

Table 15.1. (*cont.*)

HCMV[a]	HHV-6[a]	HHV-7[a]	HCMV gene family or function[b]	Selected references[c]
UL49†	U33	U33		
UL50†	U34	U34	(core) Inner nuclear membrane protein; nuclear egress of capsids in MCMV; virion protein? (NEMP)	(Muranyi *et al.*, 2002; Varnum *et al.*, 2004)
UL51†	U35	U35	(core) DNA packaging; terminase-binding? (TERbp)	(Krosky *et al.*, 2000)
UL52†	U36	U36	(core) Capsid transport? (CNTP)	(Krosky *et al.*, 2000)
UL53†	U37	U37	(core) Nuclear matrix protein; nuclear egress of capsids in MCMV (NELP)	(Dal Monte *et al.*, 2002; Muranyi *et al.*, 2002)
UL54†	U38	U38	(core) DNA polymerase catalytic subunit (POL)	(Ihara *et al.*, 1994; Loregian *et al.*, 2004a; Loregian *et al.*, 2004b) [(Yoon *et al.*, 2004)]
UL55†	U39	U39	(core) Virion glycoprotein B (gB); homomultimers; heparan-binding, entry and signaling. [roseolavirus IE gene]	(Jarvis *et al.*, 2004; Lopper and Compton, 2004; Strive *et al.*, 2004; Wang *et al.*, 2003) [(Menegazzi *et al.*, 1999; Mirandola *et al.*, 1998)]
UL56†	U40	U40	(core) Terminase subunit?; binds to DNA packaging motif, exhibits nuclease activity (TER2).	(Bogner, 2002; Krosky *et al.*, 1998; Scheffczik *et al.*, 2002; Scholz *et al.*, 2003)
UL57†	U41	U41	(core) Single-stranded DNA-binding protein (SSB) [roseolavirus IE gene]	(Anders and Gibson, 1988; Kemble *et al.*, 1987) [(Menegazzi *et al.*, 1999; Rotola *et al.*, 1998)]
oriLyt†	*oriLyt*	*oriLyt*	*DNA replication origin for productive infection (cis-acting) Position conserved, sequence diverged between HCMV and roseolaviruses.*	(Anders *et al.*, 1992; Masse *et al.*, 1992; Prichard *et al.*, 1998) [(Dewhurst *et al.*, 1993; Dykes *et al.*, 1997; Krug *et al.*, 2001; Stamey *et al.*, 1995; van Loon *et al.*, 1997)]
UL69¥	U42	U42	(core) Regulatory protein; tegument protein; contributes to cell cycle block; exhibits nucleocytoplasmic shuttling; tegument protein [roseolavirus IE gene]	(Hayashi *et al.*, 2000; Lischka *et al.*, 2001) [(Menegazzi *et al.*, 1999; Mirandola *et al.*, 1998)]
UL70†	U43	U43	(core) Component of DNA helicase-primase; primase homology (HP2)	(McMahon and Anders, 2002)
UL71†	U44	U44	(core) Tegument protein; cytoplasmic egress? (CEF1)	
UL72	U45	U45	(core) Deoxyuridine triphosphatase homologue (enzymatically inactive), virion protein (dUTPase);	(Caposio *et al.*, 2004; Varnum *et al.*, 2004)
UL73†	U46	U46	(core) Virion glycoprotein N (gN); complexes with gM; entry	(Dal Monte *et al.*, 2004; Mach *et al.*, 2000; Mach *et al.*, 2005)
UL74¶	U47	U47	Virion glycoprotein O (gO); complexes with gH and gL in HCMV and roseolaviruses	(Hobom *et al.*, 2000; Huber and Compton, 1998; Theiler and Compton, 2002) [(Mori *et al.*, 2004)]
UL75†	U48	U48	(core) Virion glycoprotein H (gH); complexes with gL and gO; entry	(Baldwin *et al.*, 2000; Hobom *et al.*, 2000) [(Santoro *et al.*, 2003)]
UL76†	U49	U49	(core) Virion-associated regulatory protein	(Wang *et al.*, 2004b)
UL77†	U50	U50	(core) Portal capping protein; role in DNA packaging (PCP)	
UL78	U51	U51	GPCR family; putative chemokine receptor	(Michel *et al.*, 2005) [(Menotti *et al.*, 1999; Milne *et al.*, 2000)]
UL79†	U52	U52		
UL80†	U53	U53	(core) Protease (N terminus) and capsid assembly (scaffold) protein (C terminus) (PR) [HHV-7 IE gene]	(Gibson, 1996; Kim *et al.*, 2004; Plafker and Gibson, 1998) [(Menegazzi *et al.*, 1999; Tigue *et al.*, 1996)]
UL80.5†	U53.5	U53.5	(core) Capsid assembly (scaffold) protein (AP)	(Casaday *et al.*, 2004; Oien *et al.*, 1997; Wood *et al.*, 1997)

(*cont.*)

Table 15.1. (*cont.*)

HCMV[a]	HHV-6[a]	HHV-7[a]	HCMV gene family or function[b]	Selected references[c]
UL82[¥]	U54	U54	UL82 family; tegument phosphoprotein (pp71; upper matrix protein); virion transactivator; ND10 localized; degrades Rb	(Bresnahan and Shenk, 2000; Hofmann *et al.*, 2002; Ishov *et al.*, 2002; Kalejta and Shenk, 2003)
UL83			UL82 family; major tegument phosphoprotein (pp65; lower matrix protein); suppresses interferon response	(Abate *et al.*, 2004)
UL84[†]	U55	U55	Role in organizing DNA replication; exhibits nucleocytoplasmic shuttling; binds IE2	(Colletti *et al.*, 2004; Colletti *et al.*, 2005; Xu *et al.*, 2004a; Xu *et al.*, 2004b)
UL85[†]	U56	U56	(core) Component of capsid triplexes (minor capsid protein; TRI2)	
UL86[†]	U57	U57	(core) Major capsid protein; component of hexons and pentons (MCP)	(Lai and Britt, 2003; Wood *et al.*, 1997) [(Littler *et al.*, 1990; Mukai *et al.*, 1995)]
UL87[†]	U58	U58		
UL88[¶]	U59	U59	(core) Tegument protein; cytoplasmic egress? (CEF2)	
UL89 × ***1***[†]	U66 × 1	U66 × 1	(core) Terminase ATPase subunit; inhibition by antiviral compounds (TER1)	(Bogner, 2002; Buerger *et al.*, 2001; Krosky *et al.*, 1998; Scheffczik *et al.*, 2002)
UL89 × *2*	U66 × 2	U66 × 2		
UL90[†]				
UL91[†]	U62	U62		
UL92[†]	U63	U63		
UL93[†]	U64	U64	(core) Tegument protein; capsid transport? (CTTP)	(Wing and Huang, 1995)
UL94[†]	U65	U65	(core) Tegument protein; binds single-stranded DNA; cytoplasmic egress? (CETPbp) [HHV-6 IE gene]	(Wing and Huang, 1995; Wing *et al.*, 1998; Wing *et al.*, 1996) [(Mirandola *et al.*, 1998)]
UL95[†]	U67	U67	(core) encapsidation chaperone protein? (ECP)	(Wing and Huang, 1995)
UL96[†]	U68	U68	Tegument protein	(Wing and Huang, 1995)
UL97[¶]	U69	U69	(core) Viral serine-threonine protein kinase; tegument protein; phosphorylates ganciclovir; inhibited by maribavir; roles in DNA synthesis, DNA packaging and nuclear egress; mimics cdc2/CDK1 (VPK)	(Baek *et al.*, 2004; Kawaguchi *et al.*, 2003; Krosky *et al.*, 2003a; Krosky *et al.*, 2003b; Talarico *et al.*, 1999; Wolf *et al.*, 2001; Wolf *et al.*, 1998) [(Ansari and Emery, 1999; Manichanh *et al.*, 2001; Michel and Mertens, 2004)]
UL98[†]	U70	U70	(core) Deoxyribonuclease (NUC)	(Gao *et al.*, 1998; Wing and Huang, 1995)
UL99[†]	U71	U71	(core) Myristylated tegument phosphoprotein pp28; cytoplasmic egress tegument protein CETP)	(Britt *et al.*, 2004; Jones and Lee, 2004; Sanchez *et al.*, 2000b; Silva *et al.*, 2005; Silva *et al.*, 2003; Wing and Huang, 1995)
UL100[†]	U72	U72	(core) Virion glycoprotein M (gM); complexes with gN; entry	(Mach *et al.*, 2000; Mach *et al.*, 2005)
	U73	U73	oriBP, binds to roseolavirus oriLyt; helicase [roseolavirus IE gene]	[(Inoue and Pellett, 1995; Krug *et al.*, 2001; Menegazzi *et al.*, 1999; Mirandola *et al.*, 1998)]
UL102[†]	U74	U74	(core) Component of DNA helicase-primase (HP3)	(McMahon and Anders, 2002)
UL103[¶]	U75	U75	(core) Tegument protein; nuclear egress? (EEP)	
UL104[†]	U76	U76	(core) Portal protein; DNA encapsidation (PORT)	(Dittmer and Bogner, 2005; Komazin *et al.*, 2004)
UL105[†]	U77	U77	(core) Component of DNA helicase-primase; helicase homology (HP1)	(McMahon and Anders, 2002; Smith *et al.*, 1996)
UL108[¶]				
UL111A			Viral interleukin 10 (vIL-10)	(Chang *et al.*, 2004; Kotenko *et al.*, 2000; Lockridge *et al.*, 2000; Spencer *et al.*, 2002)
UL112[¥]	U79 × 1–2	U79 × 1–2	Transcriptional activation, orchestration of	(Ahn *et al.*, 1999; Li *et al.*, 1999; Penfold and
UL113[¥]	U79 × 3	U79 × 3	DNA replication	Mocarski, 1997) [(Taniguchi *et al.*, 2000)]

Table 15.1. (*cont.*)

HCMV[a]	HHV-6[a]	HHV-7[a]	HCMV gene family or function[b]	Selected references[c]
UL114[¶]	U81	U81	(core) Uracil-DNA glycosylase; roles in excision of uracil from DNA and temporal regulation of DNA replication (UNG) [HHV-6 IE gene]	(Courcelle *et al.*, 2001; Prichard *et al.*, 1996) [(Rotola *et al.*, 1998)]
UL115[†]	U82	U82	(core) Virion glycoprotein L (gL); complexes with gH and gO; entry	(Britt and Mach, 1996; Huber and Compton, 1999; Kaye *et al.*, 1992; Milne *et al.*, 1998) [(Mori *et al.*, 2004; Mori *et al.*, 2003b)]
UL116			Putative membrane glycoprotein	
	U83		CC chemokine	(French *et al.*, 1999; Luttichau *et al.*, 2003; Zou *et al.*, 1999)
UL117[¥]	U84	U84		
UL119		U85[d]	IgG Fc-binding membrane glycoprotein related to OX-2; virion glycoprotein	(Atalay *et al.*, 2002; Varnum *et al.*, 2004)
UL120			UL120 family; putative membrane glycoprotein	
UL121			UL120 family; putative membrane glycoprotein	
UL122[†]	U86	U86	Immediate early transactivator (IE2); interacts with transcriptional machinery; repression via specific DNA-binding activity	(Barrasa *et al.*, 2005; Heider *et al.*, 2002; Lee and Ahn, 2004; Marchini *et al.*, 2001; Sanchez *et al.*, 2002) [(Gravel *et al.*, 2003; Papanikolaou *et al.*, 2002)]
UL123[¥]	U90[d]	U90[d]	Immediate early transactivator (IE1); enhances activation by IE2; indirect effect on transcription machinery; disrupts ND10 [roseolavirus IE gene]	(Ahn and Hayward, 2000; Gawn and Greaves, 2002; Greaves and Mocarski, 1998; Lee and Ahn, 2004; Mocarski *et al.*, 1996; Nevels *et al.*, 2004; Reinhardt *et al.*, 2005) [(Gravel *et al.*, 2003; Menegazzi *et al.*, 1999; Mirandola *et al.*, 1998; Nikolaou *et al.*, 2003)]
UL124	U91[d]	U91[d]	Membrane glycoprotein, latent protein [HHV-6 IE gene]	(Kondo *et al.*, 1996; Landini *et al.*, 2000) [(Rotola *et al.*, 1998)]
	U94	U94	Parvovirus Rep protein homologue; binds to a transcription factor and single-stranded DNA; latent protein	[(Dhepakson *et al.*, 2002; Rotola *et al.*, 1998);]
	U95	U95	US22 family; HHV-6 IE gene related to MCMV IE2; positional to HCMV IRS1/TRS1	(Takemoto *et al.*, 2001)
	U100	U100	Virion glycoprotein Q; complexed with gH and gL	(Mori *et al.*, 2003a; Mori *et al.*, 2003b)
UL128			Putative secreted protein; putative CC chemokine; endothelial cell tropism	(Akter *et al.*, 2003; Hahn *et al.*, 2004)
UL129[¶]				
UL130			Putative secreted protein	(Akter *et al.*, 2003; Hahn *et al.*, 2004)
UL131A			Putative secreted protein	(Akter *et al.*, 2003; Hahn *et al.*, 2004)
UL132[¶]			Virion glycoprotein	(Varnum *et al.*, 2004)
UL148			Putative membrane glycoprotein	
UL147A			Putative membrane protein	
UL147			UL146 family; putative secreted glycoprotein; putative CXC chemokine	(Penfold *et al.*, 1999; Prichard *et al.*, 2001)
UL146			UL146 family; secreted glycoprotein; CXC chemokine	(Penfold *et al.*, 1999; Prichard *et al.*, 2001
UL145				
UL144			Membrane glycoprotein; TNF receptor homologue; Regulate lymphocytes via BTLA	(Sedy *et al.*, 2005) (Ware, personal communication)
UL142			UL18 family; putative membrane glycoprotein; MHC-I homologue	(Davison *et al.*, 2003)

(*cont.*)

Table 15.1. (*cont.*)

HCMV[a]	HHV-6[a]	HHV-7[a]	HCMV gene family or function[b]	Selected references[c]
UL141			UL14 family; membrane glycoprotein; inhibits NK cell cytotoxicity by downregulating CD155	(Tomasec *et al.*, 2005)
UL140			Putative membrane protein	
UL139			Putative membrane glycoprotein	
UL138			Putative membrane protein	
UL136			Putative membrane protein	
UL135			Putative secreted protein	
UL133			Putative membrane protein	
UL148A			Putative membrane protein	
UL148B			Putative membrane protein	
UL148C			Putative membrane protein	
UL148D			Putative membrane protein	
UL150			Putative secreted protein	
IRS1			US22 family; immediate early transcriptional activator; tegument protein; blocks shut-off of host protein synthesis; virion protein [positional to roseolavirus U95]	(Child *et al.*, 2002; Romanowski *et al.*, 1997; Romanowski and Shenk, 1997; Stasiak and Mocarski, 1992); Child *et al.*, 2004
US1			US1 family	
US2			US2 family; membrane glycoprotein; degradation of MHC-I and, possibly, MHC-II	(Gewurz *et al.*, 2001; Tortorella *et al.*, 2000)
US3			US2 family; immediate early gene; membrane glycoprotein; inhibits processing and transport of MHC-I and MHC-II	(Gewurz *et al.*, 2001; Hegde *et al.*, 2002; Misaghi *et al.*, 2004; Tortorella *et al.*, 2000; Zhao and Biegalke, 2003)
US6			US6 family; putative membrane glycoprotein; inhibits TAP-mediated ER peptide transport	(Gewurz *et al.*, 2001; Hewitt *et al.*, 2001; Tortorella *et al.*, 2000; Ulbrecht *et al.*, 2003)
US7			US6 family; membrane glycoprotein	(Huber *et al.*, 2002)
US8			US6 family; membrane glycoprotein; binds to MHC-I	(Tirabassi and Ploegh, 2002)
US9			US6 family; membrane glycoprotein; cell-to-cell spread	(Huber *et al.*, 2002; Maidji *et al.*, 1998)
US10			US6 family; membrane glycoprotein; delays trafficking of MHC-I	(Furman *et al.*, 2002)
US11			US6 family; membrane glycoprotein; selective degradation of MHC-I	(Gewurz *et al.*, 2001; Tirosh *et al.*, 2005; Tortorella *et al.*, 2000)
US12			US12 family; putative multiple transmembrane protein	
US13¶			US12 family; putative multiple transmembrane protein	
US14			US12 family; putative multiple transmembrane protein	
US15			US12 family; putative multiple transmembrane protein	
US16*			US12 family; putative multiple transmembrane protein; temperance for ECs	
US17			US12 family; putative multiple transmembrane protein	
US18			US12 family; putative multiple transmembrane protein	(Guo and Huang, 1993)
US19*			US12 family; putative multiple transmembrane protein; temperance for ECs	(Guo and Huang, 1993)
US20			US12 family; putative multiple transmembrane protein	(Guo and Huang, 1993)

Table 15.1. (*cont.*)

HCMV[a]	HHV-6[a]	HHV-7[a]	HCMV gene family or function[b]	Selected references[c]
US21			US12 family; putative multiple transmembrane protein	
US22	DR1 × 1 DR1 × 2	DR1 DR2	US22 family; tegument protein; released from cells (HHV-6 DR1 homologue)	(Adair *et al.*, 2002)
US23[¶]			US22 family; tegument protein	(Varnum *et al.*, 2004)
US24			US22 family; tegument protein	(Varnum *et al.*, 2004)
US26[¥]	DR6 × 1 DR6 × 2	DR6 DR7	US22 family (HHV-6 DR-6 homologue)	[(Doniger *et al.*, 1999)]
US27			GPCR family; virion glycoprotein	(Fraile-Ramos *et al.*, 2002; Varnum *et al.*, 2004)
US28			GPCR family; membrane protein; broad spectrum CC and CX3C chemokine receptor; mediates cellular activation and migration	(Billstrom *et al.*, 1999; Casarosa *et al.*, 2001; Gao and Murphy, 1994; Hertel and Mocarski, 2004; Kledal *et al.*, 1998; Melnychuk *et al.*, 2004; Neote *et al.*, 1993; Streblow *et al.*, 1999; Vieira *et al.*, 1998)
US29			Putative membrane glycoprotein, necessary in RPE cells.	
US30*			Putative membrane glycoprotein; temperance for fibroblasts	
US31			US1 family	
US32			US1 family	
US34			Putative secreted protein	
US34A			Putative membrane protein	
TRS1[¶]			US22 family; immediate early transcriptional activator; tegument protein involved in capsid assembly; blocks shut-off of translation [positional to roseolavirus U95]	(Child *et al.*, 2002; Romanowski *et al.*, 1997; Romanowski and Shenk, 1997; Stasiak and Mocarski, 1992); Child *et al.*, 2004

[a] Commonly annotated ORFs of HCMV, together with selected ORFs of HHV-6 (A or B) and HHV-7. Emphasis is on ORFs that are conserved between these viruses. General references to gene mapping, homologies and annotation (Cha *et al.*, 1996; Chee *et al.*, 1990; Davison *et al.*, 2003; Dolan *et al.*, 2004; Dominguez *et al.*, 1999b; Gompels *et al.*, 1995; Nicholas, 1996). Functional dissection of HCMV (Dunn *et al.*, 2003b; Yu *et al.*, 2003) by mutagenesis showing replication better than wild type (*), indistinguishable from wild type (normal type) or one of three broad growth deficient phenotypes: failure to replicate (bold †), very poor replication (bold ¥), slight replication defect (bold ¶) when viruses are assayed in human fibroblasts. Mutations of putative HCMV ORFs UL60 and UL61 are interpreted as disruption of HCMV oriLyt (Masse *et al.*, 1992) rather than a protein-coding ORF.

[b] Betaherpesvirus-common genes in italics, and include 40 herpesvirus core functions (see Chapters 2 and 3). Gene family, characteristics and functional information are provided for HCMV where a blank indicates the absence of functional information.

[c] References listed are to gene function where known in HCMV, with those specific to HHV-6 indicated within []; see Chapter 14 for additional references to mapping and physical characteristics. General reference for HCMV structural proteins is (Baldick and Shenk, 1996).

[d] Positional homologue.

viral mutants accentuate these effects. The herpesvirus core gene products, discussed elsewhere (Chapter 4), play roles in replication. A majority, 33, core genes (UL44, UL46, UL48, UL48A, UL50–UL57, UL70, UL71, UL73, UL75–UL77, UL80, UL80.5, UL85, UL86, UL89, UL93–UL95, UL98, UL99, UL100, UL102, UL104, UL105, UL115) are absolutely essential for HCMV replication. Mutants deficient in one (UL69) exhibit severely reduced replication efficiency (Hayashi *et al.*, 2000) and mutants in five (UL47, UL88, UL97, UL103, UL114) exhibit slightly reduced replication efficiency (Table 15.1) that for UL97 or UL114 can be dependent on the fibroblast cell cycle state (Courcelle *et al.*, 2001; Prichard *et al.*, 1996, 1999; Wolf *et al.*, 2001). Only two core genes, the ribonucleotide reductase homologue (HCMV UL45) and the dUTPase homologue (HCMV UL72), lack any detectable role in replication (Hahn *et al.*, 2002, 2003). Neither of these betaherpesvirus homologues conserve key motifs for enzymatic activity that are found in other herpesviruses leaving an open question as to function.

Many of the 30-odd betaherpesvirus-specific genes investigated individually (Bresnahan and Shenk, 2000;

Greaves and Mocarski, 1998; Marchini *et al.*, 2001) or systematically (Dunn *et al.*, 2003b; Yu *et al.*, 2003) have been found to play some role in viral replication. Ten are essential for HCMV replication in fibroblasts (UL32, UL34, UL49, UL79, UL84, UL87, UL91, UL92, UL96, UL122), six (UL28, UL82, UL112–113, UL117, UL123, US26) have a strong impact on growth and six (UL29, UL31, UL35, UL38, UL74, TRS1) have only a slight impact on growth. While the two systematic mutagenesis efforts (Dunn *et al.*, 2003b; Yu *et al.*, 2003) did not completely agree on the phenotype associated with each gene, there was a remarkable consistency given that different laboratory strains of virus were analyzed and each was completed independently. Although characterization has not continued for most genes, 11 betaherpesvirus-specific genes (UL23, UL24, UL27, UL33, UL36, UL37 × 3, UL43, UL78, UL124, IRS1, US22) play no detectable role in fibroblasts (Dunn *et al.*, 2003b). These include a tropism factor encoded by UL24, which is necessary for replication in endothelial cells (Dunn *et al.*, 2003b), as well as UL36, which encodes the viral inhibitor of caspase 8 activation (vICA), a potent cell death suppressor (McCormick *et al.*, 2003; Skaletskaya *et al.*, 2001) that influences infected cell survival in the presence of inducers of cell death. The role of the two cell death suppressors encoded by HCMV, vICA and viral mitrochondrial inhibitor of apoptosis (vMIA), appear to overlap in protection from cell death during infection (McCormick *et al.*, 2005) despite evidence that only vMIA was important in strain AD169 var ATCC (Reboredo *et al.*, 2004).

Genetic analysis has only begun in the roseolaviruses due to the relative intractability of these viruses to molecular genetic manipulation. The one insertion mutant of HHV-6B that has been reported, disrupting betaherpesvirus-specific U3-U7 (homologues of HCMV UL24, UL27 and UL28), replicates normally (Kondo *et al.*, 2003a), consistent with the behavior of individual gene mutants in HCMV homologues (Dunn *et al.*, 2003b).

One long-standing unique characteristic of the HCMV genome is the presence of gene families (Chee *et al.*, 1990; Dolan *et al.*, 2004), as described in Chapter 14, which extend to all betaherpesviruses (Dominguez *et al.*, 1999a; Gompels and Macaulay, 1995; Nicholas, 1996). The US22 family is the largest and most highly conserved of these families, and includes a great many genes that are dispensable for replication but that play roles in cell survival, cell tropism and pathogenesis (Dunn *et al.*, 2003b; McCormick *et al.*, 2003; Menard *et al.*, 2003; Skaletskaya *et al.*, 2001). HCMV encodes 13 US22 family members (Chee *et al.*, 1990) whereas roseolaviruses encode ten, nine of which are conserved in sequence or genomic location (Table 15.1). Cell tropism appears to be influenced by different members of the US22 family in different viruses. In HCMV, these are involved in tropism for particular cell types or in tempering viral replication in certain cell types (Dunn *et al.*, 2003b). For example, US22 family member UL23 has been implicated as a temperance factor for HCMV growth in fibroblasts. Other identified temperance genes (Table 15.1) are not in the US22 family and are not conserved in betaherpesviruses. The US22 family member UL24 has been implicated as a tropism factor in endothelial cells (Dunn *et al.*, 2003b) and TRS1 protein blocks activation of protein kinase R in the interferon response (Child *et al.*, 2004). Many additional genomic regions have been implicated in the control of cell tropism (Bolovan-Fritts and Wiedeman, 2002; Brown *et al.*, 1995; Cha *et al.*, 1996; Dunn *et al.*, 2003b; Gerna *et al.*, 2003; Hahn *et al.*, 2004; Hertel *et al.*, 2003; Jahn *et al.*, 1999; Kahl *et al.*, 2000; Mocarski *et al.*, 1993; Riegler *et al.*, 2000; Sinzger and Jahn, 1996; Sinzger *et al.*, 2000) but none of these has been associated with specific betaherpesvirus-conserved genes or US22 family members. Insights into tropism have been derived from studies on animal models, primarily MCMV (Brune *et al.*, 2001; Cavanaugh *et al.*, 1996; Grzimek *et al.*, 1999; Manning *et al.*, 1992; Menard *et al.*, 2003; Morello *et al.*, 1999), where evaluation in cell culture as well as in the natural host is experimentally tractable. Using MCMV, tropism for endothelial cells and macrophages has been studied in cultured cells as well as in mice and is influenced by herpesvirus core (RR1) as well as by betaherpesvirus-common (US22 family) gene products (Brune *et al.*, 2001; Hanson *et al.*, 2001; Menard *et al.*, 2003). Betaherpesvirus-conserved tegument-associated US22 family member M36, which encodes vICA similar to HCMV UL36 (McCormick *et al.*, 2003), as well as other US22 family members whose precise homologues cannot be assigned, is a specific determinant of cell tropism for macrophages but not for fibroblasts or endothelial cells (Hanson *et al.*, 2001; Menard *et al.*, 2003). The US22 family member M43 appears to influence viral growth in macrophage-like cells as well as immortalized fibroblasts, but not in primary cells of either cell type (Menard *et al.*, 2003). Unfortunately, the two US22 family members contributing to HCMV endothelial cell tropism (UL24) or temperance (UL23) (Dunn *et al.*, 2003b) have not been found to play a role in tropism for endothelial cells or macrophages when MCMV has been studied (Menard *et al.*, 2003). Also, MCMV studies have thus far not given insights into HCMV (Dunn *et al.*, 2003b; Hahn *et al.*, 2002) such that HCMV RR1 and particular US22 family members implicated in MCMV cell tropism do not exhibit a similar impact on HCMV tropism. Thus, betaherpesvirus-conserved genes have not yet been implicated in common pathways of tropism even though a large proportion of the gene products in betaherpesviruses such as HCMV as well as MCMV appear to be involved in

modulating the host response to infection (Mocarski, 2002; 2004). All betaherpesviruses encode homologues of HCMV UL33 and UL78, seven transmembrane spanning receptors as well as chemokine receptor homologues. Viruses like HCMV and MCMV immunomodulate similar cellular processes, however, these viruses often rely on evolutionarily distinct gene products to do so. Important immunomodulatory functions are therefore largely not reflected in the betaherpesvirus-conserved set of genes.

Discussion here will focus on HCMV, with presentation of betaherpesvirus-common functions in virion structure and entry, proceeding through regulation of gene expression, DNA synthesis, processing and packaging and, finally, maturation and egress. Because of apparent similarity in the genomic location and structure latent transcripts that have been mapped in HCMV (Kondo *et al.*, 1996) and HHV-6B (Kondo *et al.*, 2002b, 2003b), the chapter will conclude with a brief discussion of the molecular basis of betaherpesvirus latency.

Virion structural proteins

Virion structure is highly conserved among betaherpesviruses such that over half the betaherpesvirus-common genes encode structural proteins. As shown in Table 15.1, 23 herpesvirus core genes encode HCMV structural proteins as components of the capsid (UL46, UL48A, UL85, UL86, UL104 gene products), tegument (UL45, UL47, UL48, UL69, UL71, UL72, UL76, UL77, UL88, UL93, UL94, UL95, UL97, UL99, UL103 gene products) or envelope (UL55, UL73, UL75, UL100, UL115 gene products). Many additional core genes are involved in assembly and maturation of virions (Chapter 4). Fifteen betaherpesvirus-specific genes (UL23, UL24, UL32, UL33, UL35, UL36, UL38, UL43, UL74, UL78, UL82, UL96, IRS1, US22, TRS1) encode structural proteins, localizing to the tegument or envelope.

Betaherpesvirus capsids are composed of four major and two minor herpesvirus-conserved proteins (Chapter 4). The major capsid protein (MCP, HCMV UL86 gene product), triplex monomer and dimer proteins (TRI1 and TRI2, HCMV UL46 and UL85 gene products, respectively), and the smallest capsid protein (SCP, HCMV UL48A gene product) are prominent capsid constituents, along with the portal protein (PORT, HCMV UL104 gene product), which likely constitutes one penton used for encapsidation of viral DNA as it does in alphaherpesviruses.

Most herpesvirus core structural proteins localize to the tegument. In HCMV, all core tegument proteins (HCMV UL47, UL48, UL71, UL76, UL77, UL88, UL93–UL97, UL99, UL103 gene products) play some role in replication (Table 15.1), although four, including a protein that likely binds to the largest tegument protein, the homologue of a cytoplasmic egress facilitator, the viral serine-threonine protein kinase (VPK) and a homologue of an encapsidation and egress protein (HCMV UL47, UL88, UL97 and UL103 gene products, respectively), have only a slight impact on viral growth. VPK apparently acts in tandem with host cell cycle kinases to regulate replication events across a spectrum depending on the state of the host cell (Krosky *et al.*, 2003a; Prichard *et al.*, 1999; Wolf *et al.*, 2001) and is dispensable for replication in rapidly dividing host cells where its role is presumably redundant with host protein kinases, possibly as a cdk1 or cdk2 analogue (Kawaguchi *et al.*, 2003). The portal capping protein (HCMV UL77 gene product) is considered part of the tegument and is essential for replication. By analogy with HSV-1, UL48 may be involved in entry (Ogawa-Goto *et al.*, 2002) and others may be involved in egress (Chapter 4). UL76 protein localizes in a pattern that suggests it may be involved in regulating events immediately following infection or during maturation (Wang *et al.*, 2004b). HCMV UL99 protein (pp28) is an abundant, small myristolated tegument protein that localizes to the cytoplasm and is involved in the late stages of virion egress (Jones and Lee, 2004; Silva *et al.*, 2003). If information from alphaherpesviruses is predictive, UL99 protein may be expected to interact with other tegument proteins, such as the UL94 and UL95 gene products, as well as with the gM:gN glycoprotein complex. Investigation of tegument protein activities and capsid–tegument or tegument–envelope interactions remain important areas for experimental investigation.

A bulk of betaherpesvirus-specific structural proteins also localize to the virion tegument (Table 15.1). The most abundant include two UL82 family members, pp65 (lower matrix protein), the UL83 gene product and is the most abundant HCMV tegument protein, and pp71 (upper matrix protein), UK82 gene product, as well as pp150 (large matrix phosphoprotein), UL32 gene product, and pp28 (myristylated tegument phosphoprotein), the UL99 gene product pp65 is a major constituent of dense bodies that are produced during HCMV replication. The abundance of UL82 family members varies in other betaherpesviruses. (Gibson, 1996) as well as several minor tegument proteins. The most abundant HCMV tegument protein (pp65, upper matrix protein, UL83 gene product), a UL82 family member, is not conserved in roseolaviruses. HCMV UL82 encodes pp71, the virion transactivator (Liu and Stinski, 1992) that exhibits an MOI-dependent role in replication (Bresnahan and Shenk, 2000). A minor HCMV tegument protein encoded by UL35 interacts with the pp71 and co-stimulates transcription (Liu and Biegalke, 2002; Schierling *et al.*, 2004, 2005). HCMV UL32 encodes pp150, an essential tegument

protein (Dunn *et al.*, 2003b) that interacts with capsids (Baxter and Gibson, 2001) and localizes to nuclear and cytoplasmic compartments (Sampaio *et al.*, 2005) where it may be involved in virion assembly, maturation or egress. Conserved US22 family members (HCMV genes UL23, UL24, UL36, UL43, IRS1, US22, TRS1) all encode tegument proteins (Table 15.1). These include the UL24 tropism factor as well as UL36, cell death suppressor (McCormick *et al.*, 2003; Skaletskaya *et al.*, 2001), as discussed in Chapter 21.

The most abundant, and functionally important envelope glycoproteins found in HCMV virions, gB, gH:gL and gM:gN, are all herpesvirus core functions. Betaherpesviruses have not yet been found to encode any unique glycoproteins that control viral attachment or entry such as gD in HSV-1 or gp220/350 in EBV. In betaherpesviruses, as well as gammaherpesviruses, the gH:gL complex may be modified by a third component. These variants may influence cell type specificity as has been characterized best in the gammaherpesvirus EBV (Chapter 23). In both HCMV and roseolaviruses, a betaherpesvirus-specific protein, gO, may associate with this complex (Huber and Compton, 1998; Mori *et al.*, 2004), and, in roseolaviruses a separate glycoprotein that is not conserved in HCMV, gQ, may be a component of gH:gL complexes (Mori *et al.*, 2003b). Functional studies on gM:gN have been reported (Mach *et al.*, 2005), suggesting that this most abundant complex in virion envelopes (Varnum *et al.*, 2004) is critical for being involved in some step in entry. Other membrane proteins such as UL33, a ligand-independent seven-transmembrane-spanning signaling protein (Casarosa *et al.*, 2003), UL78, and UL38 may be associated with the virion envelope (Varnum *et al.*, 2004), but viral replication in fibroblasts is not influenced in their absence (Dunn *et al.*, 2003b).

Entry into host cells

The mechanisms of betaherpesvirus binding to the cell surface and of fusion-mediated penetration have been extensively studied but the details of these processes are still incomplete (Chapters 16 and 46). Herpesvirus core glycoproteins appear to play the critical roles in entry (Fig. 15.1), relying on gB, gH:gL and gM:gN (Chapter 4). HCMV entry into fibroblasts is via direct fusion at the cell surface (Compton *et al.*, 1992) and possibly via endocytosis in other cell types (see Chapter 16). Roseolaviruses employ direct penetration as well as endocytic pathways of entry (Mori *et al.*, 2002). Entry of betaherpesviruses appears to involve the interaction of these envelope glycoproteins with a number of distinct receptors, and is initiated by a common use of the cell surface proteoglycan heparan sulfate (Compton *et al.*, 1993; Conti *et al.*, 2000; Neyts *et al.*, 1992). The core protein gB from both HCMV (Compton *et al.*, 1993; Kari and Gehrz, 1993) and roseolaviruses (Conti *et al.*, 2000; Secchiero *et al.*, 1997) binds heparan sulfate. This initial contact is followed by interactions with additional receptors. In HCMV, a variety of cell surface proteins have been proposed to play roles in the subsequent steps in attachment (Chapter 16), although none is yet widely accepted. In addition to the role of receptors as entry mediators, HCMV binding to cells induces signaling cascades (Evers *et al.*, 2004) that activate NF-κB (Yurochko and Huang, 1999; Yurochko *et al.*, 1995), mitogen-activated protein kinase (Johnson *et al.*, 2000), phosphotidylinositol 3 kinase (Johnson *et al.*, 2001), and toll-like pathways (Compton *et al.*, 2003). gB binding seems sufficient for induction of some pathways (Boehme *et al.*, 2004; Boyle *et al.*, 1999; Wang *et al.*, 2003; Yurochko *et al.*, 1997). HCMV gB has been shown to utilize the EGF receptor as well as integrins as entry mediators (Feire *et al.*, 2004; Wang *et al.*, 2003). Signaling may play a critical role in conditioning the cell for viral replication following entry (Wang *et al.*, 2003). In HHV-6A or HHV-6B, CD46 (Santoro *et al.*, 1999) is a critical cellular receptor and interacts with gH-containing glycoprotein complexes (Mori *et al.*, 2004; Mori *et al.*, 2003b; Santoro *et al.*, 2003). Entry of HHV-7 into T cells relies on CD4 (Lusso *et al.*, 1994). Thus, except for initial contact with proteoglycan and the apparent use of core glycoproteins, betaherpesvirus attachment and entry mechanisms appear diverse at the level of current knowledge.

The same set of envelope glycoproteins (gB, gH:gL and gM:gN) have been implicated in fusion between the envelope and the cell membrane (Compton, 2004). HCMV gB and gH have been the most extensively studied for fusion (Baldwin *et al.*, 2000; Feire *et al.*, 2004; Lopper and Compton, 2004; Navarro *et al.*, 1993; Tugizov *et al.*, 1994; Wang *et al.*, 2003). HHV-6A gB is also relatively well studied (Santoro *et al.*, 2003). Recent evidence suggests core envelope glycoproteins gM and gN (gM:gN) may also be important for entry of betaherpesviruses (Mach *et al.*, 2005) despite the fact that their involvement in alphaherpesvirus entry is variable (Chapter 4). The best evidence suggests that the core envelope glycoprotein complexes formed by gB (also called gCI) or gH:gL (also called gCIII) are essential for replication in HCMV (Hobom *et al.*, 2000) because of the role these conserved glycoproteins play in fusion. The role of gH:gL modification by betaherpesvirus-specific UL74 gene product, gO (Hobom *et al.*, 2000; Huber and Compton, 1998; Theiler and Compton, 2002) remains unknown due to its relatively minor impact on viral replication (Dunn *et al.*, 2003b). In HHV-6B, gH:gL modified by either the U47 gene product gO (Mori *et al.*, 2004) or the U100 gene product gQ (Mori *et al.*, 2003a) (Table 15.1) facilitate entry into cells via CD46 (Mori *et al.*, 2003b).

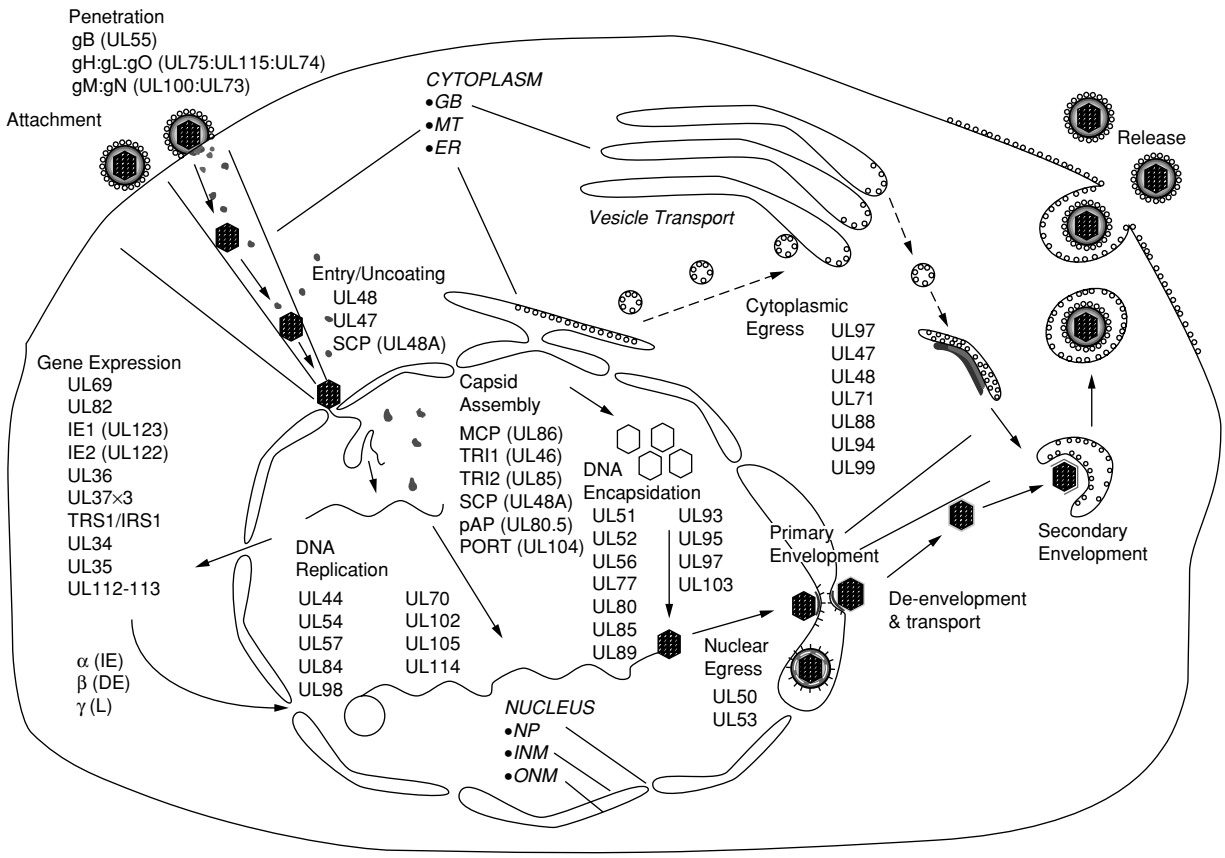

Fig. 15.1. Summary of replication functions carried out by betaherpesvirus-conserved gene products using HCMV gene designations. Major steps in productive replication are indicated along with functions (see Table 15.1) contributing to each step are listed in plain font. The entry pathway shown (black arrows) employs direct fusion at the cell surface (attachment and penetration). Entry may also follow an endocytic pathway and virion fusion with an endocytic vesicle in some betaherpesviruses (see text). Entry requires gB, gH:gL, possibly with gO, and gM:gN. HCMV UL47, UL48 and SCP gene products may mediate transport on microtubules, docking at nuclear pores and release of virion DNA into the nucleus based on work with alphaherpesviruses. One core regulatory protein (UL69) and several betaherpesvirus-specific proteins in the virion (HCMV UL82, UL36, TRS1/IRS1 gene products). expressed as IE genes (HCMV IE1, IE2, UL37 × 3 gene products) or expressed as DE genes (HCMV UL34, UL35 UL112–113 gene products) are involved in transcriptional regulation. DNA replication depends on several core proteins (HCMV UL44, UL54, UL57, UL70, UL102, UL105, UL98, UL114 gene products) as well as one betaherpesvirus-specific protein (UL84 gene product). Capsid assembly uses core functions MCP, TRI1:TRI2, SCP, PORT and pAP. Pre-formed capsids likely translocate to sites of DNA replication where several core proteins (HCMV UL51, UL56, UL77, UL80.5, UL85, UL89, UL93, UL95, UL97, UL103 gene products) are likely to be involved in encapsidation of viral DNA. Nuclear egress is likely to be controlled by HCMV UL50 and UL53 gene products, which are core proteins. Cytoplasmic egress (black arrows) and secondary (final) envelopment are controlled by core functions (HCMV UL47, UL48, UL71, UL88, UL94, UL97, UL99 gene products). Nucleocapsids are likely transported on MT and virion envelope glycoproteins follow vesicle transport to sites of final envelopment in the cytoplasm. Golgi body (GB), microtubules (MT) and endoplasmic reticulum (ER) are identified in the cytoplasm, and nuclear pores (NP), inner nuclear membrane (INM) and outer nuclear membrane (ONM) are identified in the nucleus. The cellular vesicle transport pathway from the ER to GB is also designated (dashed grey arrow). The core functions UL45 (RR1 homologue) and UL72 (dUTPase homologue) are not assigned. Functions for the betherpesvirus-common genes UL28, UL29, UL31, UL32, UL38, UL49, UL76, UL79, UL87, UL91, UL92, UL96, UL117 and US26, all of which have measurable roles in HCMV replication (Table 15.1) are not known.

The importance of the nocadozole-sensitive microtubule network in transport of HCMV nucleocapsids through the cytoplasm to the nucleus (Ogawa-Goto *et al.*, 2003) suggests that betaherpesviruses exploit normal cytoplasmic transport systems to control nucleocapsid transit through the cytoplasm. The bidirectional nature of microtubule-directed transport may allow these filaments to act as the major highways of virus particle translocation during entry as well as egress of all herpesviruses (Chapter 4). It is likely that capsid or proximal tegument proteins that are exposed following fusion mediate trafficking as well as release of the viral genome into the nucleus.

Regulation of gene expression and replication

One of the largest categories of betaherpesvirus-conserved genes encode regulatory proteins. The core homologue of the multifunctional regulator of expression found in all herpesviruses, HCMV UL69, encodes a late gene product incorporated into the tegument. This gene product regulates cell cycle progression (Hayashi *et al.*, 2000) and exhibits similarities to other herpesvirus homologues such as the capacity to shuttle between the nucleus and cytoplasm (Lischka *et al.*, 2001). In the roseolaviruses, this gene (U42) is expressed with immediate early characteristics similar to the alpha-and gammaherpesviruses (Menegazzi *et al.*, 1999; Mirandola *et al.*, 1998), although its function has not been elucidated. Although only this one regulatory protein is included in the herpesvirus core set, a number of additional virion-associated, immediate early and delayed early gene products common to all betaherpesvirus appear to carry out regulatory processes critical to replication.

HCMV UL82 encodes pp71, a virion structural protein that localizes to the nucleus soon after infection and controls transactivation of the immediate early genes (Liu and Stinski, 1992), impacting the levels of viral infection (Bresnahan and Shenk, 2000) as well as the efficiency of viral DNA transfection (Baldick *et al.*, 1997). This gene product facilitates the interaction of viral regulatory and DNA replication proteins with nuclear domain (ND) 10 regions at the start of infection (Hofmann *et al.*, 2002; Ishov *et al.*, 2002), and may possibly control cell cycle regulation via Rb degradation (Kalejta and Shenk, 2003). The UL82 gene product interacts with another betaherpesvirus-conserved tegument protein encoded by the UL35 gene, and appears to cooperate in transactivation as well as play an accessory role in maturation (Schierling *et al.*, 2004, 2005). The roseolaviruses have only a single copy of this gene and this is most closely related to HCMV UL82. Additional UL82 family members are present in many betaherpesviruses where they encode major tegument proteins, such as the UL83 gene product (major tegument phosphoprotein) of HCMV that modulates the host cell response to infection (Abate *et al.*, 2004).

HCMV and roseolaviruses encode major immediate early nuclear proteins that have been designated IE1 and IE2 (Chapter 17). This gene cluster exhibits positional, structural and functional similarities across the betaherpesviruses although the primary amino acid sequence of these two proteins diverges dramatically. These multiply spliced gene products are abundantly made in infected cells where they are the major transactivators of viral gene expression during productive infection. In HCMV, where these gene products have been studied extensively, IE2, an 86 kDa protein, is a critical regulatory gene product responsible for activation of delayed early and late genes (Heider *et al.*, 2002; Marchini *et al.*, 2001; Pizzorno *et al.*, 1991), and IE1, a 72 kDa protein, increases the potency of IE2 in the activation of viral genes and is critical for viral replication following low MOI infection (Gawn and Greaves, 2002; Greaves and Mocarski, 1998). HCMV IE2 interacts with a variety of cellular transcription factors as an adapter or modulator of the host transcription complex to activate gene expression. IE2 is a site-specific DNA binding protein that mediates autoregulatory shut-off of IE1/IE2 region transcription at late times of infection via a specific promoter element (Chapters 17 and 18). IE1 interacts with host chromatin and cellular proteins but has not been found to directly bind DNA. IE1 (Reinhardt *et al.*, 2005) and IE2 (Pizzorno *et al.*, 1991; White *et al.*, 2004) contain distinct acidic activation domains that appear to be crucial for transactivation as well as for their critical role in viral replication.

Minor immediate early proteins are also conserved as betaherpesvirus-specific gene products (Chapter 21). HCMV immediate early proteins TRS1 and IRS1 were initially recognized as regulatory proteins working in conjunction with IE1 and IE2 in activation of delayed early and late gene expression (Iskenderian *et al.*, 1996; Stasiak and Mocarski, 1992), and although virus replication is more highly dependent on TRS1 than IRS1, neither gene product is crucial for virus replication (Blankenship and Shenk, 2002; Dunn *et al.*, 2003b). Mechanistically, the function of these proteins seems focused on the interferon response (Child *et al.*, 2004), which possibly underlies all phenotypes that have been reported.

HCMV also encodes two cell death suppressors (HCMV VICA encoded by UL36 and vMIA encoded by UL37 × 1), one of which (UL36) is more obviously conserved in all betaherpesviruses examined (McCormick *et al.*, 2003), is mutated in some strains of HCMV (Skaletskaya *et al.*, 2001) and is dispensable for replication (Patterson and Shenk,

1999). HCMV UL37 × 1 is a potent inhibitor of apoptosis (Goldmacher *et al.*, 1999) also dispensable (McCormick *et al.*, 2005), that was first thought to only be conserved amongst primate cytomegaloviruses (McCormick *et al.*, 2003). It is now clear that the MCMVm 38.5 gene functions like vMIA (McCormick *et al.*, 2005). HCMV UL37×3, which was originally designated UL37 (Chee *et al.*, 1990), is an immediate early gene product that is spliced to UL37×1 to produce a number of related gene products (Adair *et al.*, 2003). An upstream exon has not been detected in other betaherpesviruses (Table 15.1). HCMV UL37×3 does not block apoptosis when expressed without UL37×1. UL37×3 and UL37×1 are dispensable for replication (Borst *et al.*, 1999; McCormick *et al.*, 2005) UL37×3 may play some role as a transactivator of viral or cellular gene expression (Hayajneh *et al.*, 2001b), possibly in conjunction with the expression of DNA replication genes (Colberg-Poley *et al.*, 1998).

Several delayed early regulatory HCMV genes are also conserved and betaherpesvirus-specific. UL112-UL113 gene products are multiply spliced and abundant delayed early nuclear proteins (Chapter 18) critical for replication (Dunn *et al.*, 2003b; Yu *et al.*, 2003) playing roles in regulation of gene expression as well as in the initial formation of viral DNA replication compartments together with IE2 (Ahn *et al.*, 1999; Penfold and Mocarski, 1997). The conserved HCMV gene UL34 encodes a number of related regulatory gene products at early and late times during infection (Biegalke *et al.*, 2004) and is an essential replication function (Dunn *et al.*, 2003b). One activity of UL34 that has been characterized is repression of US3 expression; however, the function of UL34 that is important for viral replication must lie elsewhere because US3 is itself an immediate early immunomodulatory gene that is dispensable for replication (Chapter 62). UL84 is another nuclear delayed early protein that, in HCMV, interacts with IE2 and appears crucial for viral DNA replication (Sarisky and Hayward, 1996; Xu *et al.*, 2004a) as well as in the possible modulation of IE2 transactivation activity (Gebert *et al.*, 1997).

Viral DNA synthesis and nucleotide metabolism

Betaherpesviruses have conserved the herpesvirus core set of six DNA synthesis enzymes necessary for lytic (productive) infection as well as a number of functions that, in HCMV, play roles in the initiation of DNA replication (Chapter 19). HCMV and the roseolaviruses replicate DNA in the nucleus of the cell and have a single, positionally conserved, lytic replication origin (oriLyt), located between the genes encoding homologues of HCMV UL57 and UL69. These viruses, however, have very different *cis*- as well as *trans*-acting functions involved in the initiation of DNA replication. On the one hand, HCMV has a large, complex origin region (Anders *et al.*, 1992; Masse *et al.*, 1992) with an embedded RNA transcript (Prichard *et al.*, 1998) that apparently requires transcriptional transactivators IE2 and ppUL84 for initiation (Xu *et al.*, 2004b). The dependence of HCMV oriLyt dependent replication on UL84 or IE2 varies with assay used (Sarisky and Hayward, 1996; Reid *et al.*, 2003). Other cytomegaloviruses such as MCMV share the complex origin in a similar genomic position, but do not share any obvious sequence elements that define oriLyt functional domains (Masse *et al.*, 1992). On the other hand, HHV-6A, HHV-6B and HHV-7 have a less complex oriLyt arranged as an inverted repeat region that interacts with a viral oriBP (Table 15.1) to initiate replication (Dewhurst *et al.*, 1993; Inoue and Pellett, 1995; Krug *et al.*, 2001; Stamey *et al.*, 1995; van Loon *et al.*, 1997). Roseolavirus DNA replication likely occurs in ways analogous to alphaherpesviruses (Boehmer and Nimonkar, 2003).

UL84, together with IE2, plays an essential role in HCMV DNA replication (Pari and Anders, 1993; Sarisky and Hayward, 1996) and is conserved in other betaherpesviruses. In HCMV, an UL84:IE2 complex (Spector and Tevethia, 1994) may orchestrate the sequential association of viral proteins with ND10 regions to promotes both transcription of viral genes and development of replication centers. The stepwise addition of UL112-UL113 gene products and the six core replication proteins is likely controlled by UL84 (Ahn *et al.*, 1999; Colletti *et al.*, 2004; Penfold and Mocarski, 1997; Xu *et al.*, 2004a,b). The phenotype of a UL84 mutant is consistent with a crucial role in the intermediate events of DNA replication in a way that may be similar to the established role of IE transactivators in gammaherpesvirus lytic DNA replication (Chapter 26). One poorly understood aspect of HCMV DNA replication is the function of RNA incorporated as a triple-stranded complex associated within oriLyt (Prichard *et al.*, 1998). Active transcription from this region during infection seems to be associated with DNA replication (Huang *et al.*, 1996), although transcripts arise from several different regions across the oriLyt region.

One unexplained, but interesting twist is that, despite their genome complexity, betaherpesviruses lack ancillary nucleotide metabolism functions to the extent found in most other herpesviruses. The betaherpesviruses lack a widely conserved thymidine kinase gene. Instead, VPK (HCMV UL97 gene product) acts as the nucleoside kinase for antiviral drugs such as ganciclovir. Herpesvirus core genes with homology to ribonucleotide reductase (RR1, the HCMV UL45 gene product) or deoxyuridine triphosphatase (dUTPase, the HCMV UL72 gene product) encode enzymatically inactive proteins, based on studies of the

RR1 homologue in HHV-7 (Sun and Conner, 1999), MCMV (Lembo *et al.*, 2004), HCMV (Hahn *et al.*, 2002; Patrone *et al.*, 2003) and the dUTPase homologue in HCMV (Caposio *et al.*, 2004). In HCMV, UL84 has been assigned UTPase activity (Colletti *et al.*, 2005) although it is not yet clear how this may be related to dUTPase or other activities of this gene product in viral replication.

Capsid assembly and DNA encapsidation

The basic features of capsid maturation common to all herpesviruses appear to be preserved in the betaherpesviruses based on the conservation of genes known to be involved in this process in alphaherpesviruses (Chapter 4). Capsid assembly in the nucleus of the host cell employs the herpesvirus-conserved components of the capsid shell (MCP, SCP, TRI1 and TRI2) working in conjunction with a precursor of the assembly protein (pAP, HCMV UL80.5 gene product). A protease (PR, also called assemblin,) is also required for maturation. This protein is made as a precursor (pre-PR) consisting of PR as its amino terminus fused to a longer polypeptide that contains a region that includes the pAP sequence as its carboxyl end (HCMV UL80 gene product). PR is a serine protease that self-cleaves in prePR to release PR as well as a variant of pAP, and also processes the PR, pAP, as well as all variants of pAP (Gibson, 1996). This processing results in the production of multiple forms of pAP with identical carboxyl termini. In addition, PORT (HCMV UL104 gene product) is incorporated as one penton likely to be necessary for DNA encapsidation. During infection, pre-PR must be cleaved into PR and variant AP for encapsidation to follow capsid formation but both of these proteins, as well as AP, are absent from mature, DNA-containing capsids. Maturation is regulated by phosphorylation of the participating proteins.

Once betaherpesvirus DNA has replicated in the nucleus, encapsidation is controlled by a conserved *cis*-acting element (cleavage/packaging or *pac* site) and a series of seven herpesvirus conserved *trans*-acting functions. Functional herpesvirus-conserved *pac*1 and *pac*2 signals are located near the genomic ends of HCMV, although their arrangement appears to be unique (Bogner *et al.*, 1998; Kemble and Mocarski, 1989; Mocarski *et al.*, 1987). The processes of DNA recognition, encapsidation and cleavage are all carried out by herpesvirus core functions (Fig. 15.1). Based on systematic mutagenesis (Dunn *et al.*, 2003b; Yu *et al.*, 2003) but limited functional investigation, encapsidation of viral DNA in HCMV is likely to involve the core gene products encoded by the essential UL51, UL52, UL56, UL77, UL80, UL89, UL93 and UL95 genes (Table 15.1; Fig. 15.1), with the UL56 and UL89 gene products forming the heterodimeric, benzimidizole-sensitive terminase (TER) (Krosky *et al.*, 1998; Scheffczik *et al.*, 2002). This complex probably interacts with the PORT (UL104 gene product) penton of the capsid (Dittmer and Bogner, 2005), likely in combination with a TER-associated protein (UL51 gene product) and portal capping protein (UL77 gene product). Based on studies in other herpesviruses (Chapter 4), these five proteins mediate recognition of a *pac* site on multi-genome DNA concatamers, docking with the appropriate site on a capsid, threading viral DNA into the caspid, cleavage at a *pac* site and sealing the genome into the nucleocapsid (Fig. 15.1). Also the herpesvirus core HCMV UL52 gene product may play a role in capsid transport and the UL80 gene product, which contains the maturational protease and assembly protein, are likely to be involved in encapsidation. Phosphorylation by the VPK influences encapsidation efficiency (Wolf *et al.*, 2001), although cellular enzymes may also play a role because the requirement for VPK is relaxed in dividing cells (Prichard *et al.*, 1999; Wolf *et al.*, 2001).

Maturation

Once viral DNA has been packaged, the nucleocapsid interacts with tegument proteins together with non-structural proteins in a complex, two-stage envelopment and egress process that starts in the nucleus and leads to virion release by exocytosis at the plasma membrane (Fig. 15.1). Insights into how this process proceeds are coming from studies in HCMV and MCMV, and the functions that have thus far been implicated are encoded by herpesvirus core genes (Chapter 4). The initial envelopment event occurs at the inner nuclear membrane and favors DNA-containing nucleocapsids. In betaherpesviruses, capsids lacking DNA mature, appear in the cytoplasm and accumulate as non-infectious enveloped particles (Gibson, 1996). Correct localization in the nucleus appears critical to proper egress. Based on studies on the MCMV homologues (Muranyi *et al.*, 2002), two proteins (HCMV UL50 and UL53 gene products) appear to form a nuclear egress complex on the inner nuclear membrane to control egress from the nucleus as well as disruption of the nuclear lamina. Based on studies in alphaherpesvirurses, the nuclear egress membrane protein (HCMV UL50 gene product), likely to be a type II membrane-spanning protein, would anchor the nuclear egress lamina protein (HCMV UL53 gene product), a phosphoprotein that interacts with the nuclear lamina (Mettenleiter, 2004). Protein kinases appear to play regulatory roles in nuclear egress. In HCMV, the conserved VPK influences nuclear egress (Krosky *et al.*, 2003a), and, in MCMV, host

protein kinase C has been implicated in disruption of the nuclear lamina to allow egress (Muranyi *et al.*, 2002). The nuclear egress complex may also participate in membrane fusion events at the outer nuclear membrane that are required to deposit the nucleocapsid in the cytoplasm (de-envelopment).

Evidence for final betaherpesvirus envelopment in the cytoplasm at late endosomal or Golgi body membranes has been suspected for many years (Tooze *et al.*, 1993). Certain virion tegument proteins associate with nucleocapsids during nuclear egress, but many are added in the cytoplasm where they play essential roles in maturation (Sanchez *et al.*, 2000a). Current evidence on alphaherpesviruses and betaherpesviruses suggests that a conserved cytoplasmic egress tegument protein (HCMV UL99 gene product, pp28) plays a critical role in the secondary, or final, envelopment at cytoplasmic membranes (Britt *et al.*, 2004; Jones and Lee, 2004; Silva *et al.*, 2003). This protein is modified by myristylation and palmitylation and localizes to the cytoplasmic face of cellular membranes. In alphaherpesviruses, this homologue interacts with another herpesvirus-conserved tegument protein, a homologue of the HCMV UL94 gene product, to form a complex involved in transport (Mettenleiter, 2004). Two additional tegument proteins conserved amongst herpesviruses (HCMV UL71 and UL88 gene products) may associate with cytoplasmic membranes and be involved in egress.

A critical role in final envelopment has recently been ascribed to the major tegument phosphoprotein pp150 (encoded by UL32), a betaherpesvirus-conserved function (Aucoin *et al.*, 2006).

Thus, final envelopment occurs in the cytoplasm and, as a consequence the tegument of virions includes small amounts of cellular proteins, in particular actin (Kattenhorn *et al.*, 2004; Varnum *et al.*, 2004), as well as RNAs (Greijer *et al.*, 2003; Terhune *et al.*, 2004) that appear to represent a quantitative sampling of the cytosol. Once final envelopment has occurred, the mature virion is transported inside of a vesicle to the cell surface for release by fusion of an exocytic vesicle with the plasma membrane, a process that is likely to follow cellular vesicle trafficking pathways (Fig. 15.1).

Latency

Although there are biological differences in the pathogenesis of betaherpesviruses, infections with cytomegaloviruses and roseolaviruses appear to share a common involvement of myeloid cells, particularly in the monocyte-macrophage lineage (Kondo *et al.*, 2002a; Sissons *et al.*, 2002; Mocarski *et al.*, 2005). Persistence, latency and reactivation of these viruses exhibit similarities as well as clear differences (Chapters 42 and 47). In HCMV as well as HHV-6B, latent transcripts with a common structure and genomic location have been mapped to the major IE locus (Chapter 47). These transcripts are found in bone marrow-derived granulocyte–macrophage progenitors naturally infected with HCMV (Kondo *et al.*, 1996) or in peripheral blood monocytes naturally infected with HHV-6B (Kondo *et al.*, 2002a,b). Although the genomic region encoding these transcripts also encodes the productive phase IE1 and IE2 gene products, and IE1 is a chromatin tethering protein similar to genome maintenance functions encoded by gammaherpesviruses (Reinhardt *et al.*, 2005), the major IE gene products are not encoded during natural or experimental latent infection. In both HCMV and HHV-6B, a number of novel gene products are associated with latent transcripts (Chapter 47). In HHV-6B, these transcripts may control a stage when IE1 is needed to facilitate reactivation (Kondo, 2003). Thus far, the function of novel latent gene products has not emerged from studies using cell culture models.

This analysis of the role of betaherpesvirus-conserved gene products has attempted to evaluate data generated in a wide variety of systems such that some predictions will certainly be clarified by further investigation. This chapter is intended to provide a starting point for evaluation of data on additional betaherpesvirus-conserved functions by providing a perspective on what is known about better-studied homologues.

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16

Early events in human cytomegalovirus infection

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Introduction

All viruses must deliver their genomes to host cells to initiate infection. The plasma membrane together with cell surface constituents serve as initial barriers to entry as well as the mediators that facilitate the process. This chapter will summarize what is known about the entry pathway of human cytomegalovirus, noting certain parallels and commonalities between human cytomegalovirus (HCMV) and other betaherpesviruses (see Chapter 46 for specific pathways of HHV-6 and HHV-7 entry). The roles of HCMV envelope glycoproteins and cellular receptors that control virion attachment and membrane fusion will be summarized. This chapter will also discuss the emerging role of signaling pathways in the early events in infection and examine how virus entry and innate immune activation may be coordinated.

In the simplest context, entry requires that enveloped viruses, including HCMV, HHV-6A or B and HHV-7, use virion envelope proteins to facilitate adherence to the cell surface and fusion between the virus envelope and a cellular membrane that results in the deposition of virion components into the cytoplasm. Following delivery to the cytoplasm, capsid or tegument proteins facilitate transport through the cytoplasm to and delivery of the viral genome to the nucleus in a process known as uncoating. Tegument proteins also translocate independent of the capsid to cytoplasmic or nuclear sites. For structurally complex viruses whose envelopes contain as many as 20 proteins and glycoproteins, attachment is a multi-step process typically involving more than one envelope glycoprotein interacting with a series of cell surface receptors that serve as primary receptors and coreceptors. One consequence of these virus-cell interactions based largely on information from negative strand RNA viruses is that predicted receptor-activated conformational changes in envelope glycoproteins play roles in membrane fusion. Multiple HCMV envelope glycoproteins are required to fuse membranes. Another consequence of these initial virus–host interactions may be the formation and/or delivery of bound virions to specialized membrane domains or compartments that are optimal for fusion and for activation of signal transduction into the cell. We have also recently learned that HCMV entry is accompanied by innate immune activation. This considerably heightens the complexity of the molecular events occurring during the early events in HCMV infection.

To begin a discussion of virus entry at the cellular level, one must first consider the basis of cellular tropism since receptors involved in entry are expressed on permissive cells. In the human host, HCMV causes systemic infection and exhibits a tropism for fibroblasts, endothelial cells, epithelial cells, monocytes/macrophages, smooth muscle cells, stromal cells, neuronal cells, neutrophils, and hepatocytes (Myerson *et al.*, 1984; Sinzger *et al.*, 2000). This exceptionally broad cellular tropism in the infected host is the basis of HCMV disease manifestation by this opportunist in a variety of organ systems and tissue types in the immunocompromised host. HCMV is considered to have a restricted cell tropism in vitro, however, entry into target cells is very promiscuous, since HCMV is able to bind, fuse and initiate replication in all tested vertebrate cell types. Productive in vitro replication is supported by primary fibroblast, endothelial and certain differentiated myeloid cells as well as some astrocytoma lines (Ibanez *et al.*, 1991; Nowlin *et al.*, 1991). The ability of HCMV to enter such a wide range of cells is consistent with either one broad common receptor or a number of cell-specific multiple cell specific receptors or a complex entry pathway in which a combination of both cell specific and broadly expressed cellular receptors are utilized. By contrast, both major variants of HHV-6 and HHV-7 are predominately T-lymphotropic

although HHV-6 can infect certain cells of myeloid lineage as well. HHV-7 uses CD4, a strictly T-lymphocyte expressed molecule as a receptor while HHV-6A and B use a more broadly distributed molecule CD46 as a receptor (Lusso *et al.*, 1994; Santoro *et al.*, 1999).

Cellular receptors for HCMV

It has been known for some time that HCMV initiates infection by binding to cell surface heparan sulfate proteoglycans (HSPGs) (Compton *et al.*, 1991). Engagement of HSPGs is one relatively conserved feature of herpesvirus entry pathways and is also thought to play a role in HHV-6 and HHV-7 interactions with lymphoid cells (Conti *et al.*, 2000). At least in cell culture systems, HCMV engagement of HSPGs is thought to play a crucial role in initial stage of entry by enhancing the engagement of subsequent receptors in a cascade that ultimately leads to fusion (Compton *et al.*, 1993). This hypothesis is further supported by biochemical analysis of HCMV binding, which indicates biphasic binding properties with multiple distinct affinities (Boyle and Compton, 1998).

The ability of HCMV to bind a broad range of cell types in culture has hampered efforts to identify cellular receptors using modern molecular approaches such as expression cloning. Over the past 20 years, numerous receptor candidates have been proposed, but none has been found to be absolutely necessary for infection of all susceptible cell types. These candidate receptor molecules have been selected on the basis of solid initial criteria but none have turned out to be a general entry mediator following further investigation. It is possible that each is important only in certain cell types or tissues. It also remains possible that functional redundancy masks their individual roles.

HCMV virions were initially shown to bind β_2 microglobulin (β_2m) in urine samples (Grundy *et al.*, 1988; McKeating *et al.*, 1987; McKeating *et al.*, 1986). This observation led to binding studies showing that HCMV tegument, not envelope, binds β_2m during release from cells (Grundy *et al.*, 1987a,b; McKeating *et al.*, 1987; Stannard, 1989). This β_2m–HCMV complex was then thought to associate with the alpha chain of host cell major histocompatibility complex (MHC) class I antigens (Beersma *et al.*, 1990; 1991; Browne *et al.*, 1990; Grundy *et al.*, 1987a,b). These data led to a model in which β_2m-coated HCMV bound MHC class I molecules, displacing β_2m. However, it was later determined that β_2m expression had no correlation with in vitro entry of HCMV or in vivo spread of MCMV infectivity (Beersma *et al.*, 1991; Polic *et al.*, 1996; Wu *et al.*, 1994). The demonstration that β_2m-deficient and MHC class I-deficient mice maintain full susceptiblity to MCMV infection (Polic *et al.*, 1996) provided the last piece of evidence confounding this hypothesis.

Virus-cell overlay blots identified a cell surface protein of approximately 30 kDa whose expression correlated with cells permissive for entry, suggesting that this cellular protein may be involved in HCMV entry (Nowlin *et al.*, 1991; Taylor and Cooper, 1990). This protein was later identified as annexin II, a protein that normally binds phospholipids and calcium and has membrane bridging capabilities (Wright *et al.*, 1993, 1995). Annexin II found associated with HCMV virions (Wright *et al.*, 1994, 1995). Although annexin II binds gB and enhances HCMV binding and fusion to phospholipid-containing membranes (Pietropaolo and Compton, 1997; Raynor *et al.*, 1999), cells devoid of annexin II are fully permissive for entry and initiation of infection (Pietropaolo and Compton, 1999). The role, if any, for annexin II in HCMV entry remains unknown but this protein's membrane bridging activity may enhance entry, cell–cell spread and/or maturation and egress.

CD13, or human aminopeptidase N, is a glycosylphosphatidylinositol-linked membrane protein that has also been implicated as a receptor. This hypothesis was based on that fact only human peripheral blood mononuclear cells (PBMCs) that were CD13 positive supported productive infection (Larsson *et al.*, 1998; Soderberg *et al.*, 1993a,b). This led to a more thorough study of this possibility in which CD13-specific antibodies, and chemical inhibitors of CD13 activity were both shown to inhibit HCMV binding and entry (Soderberg *et al.*, 1993a). Excitement from this report was dampened by later reports that CD13 antibodies bind to and neutralize virus before contact with cells and by the fact that entry of HCMV into CD13 depleted cells remains normal (Giugni *et al.*, 1996). More recently, an interaction between HCMV and CD13 was shown to be important in inhibition of differentiation of monocytes into macrophages suggesting this may be a strategy for interference with cellular differentiation pathways (Gredmark *et al.*, 2004).

A consideration of HCMV-induced signaling cascades led Huang and colleagues to hypothesize a role for epidermal growth factor receptor (EGFR) as a HCMV receptor (Wang *et al.*, 2003). EGFR was reported to be phosphorylated in response to HCMV and this phosphorylation event correlated with the activation of phosphatidylinositol 3-kinase (PI-3 kinase) and Akt, as well as the mobilization of intracellular Ca^{2+}. These signaling events were blocked in the presence of EGFR antibodies. In addition, chemically cross-linked virus provided evidence for a gB–EGFR interaction. A limitation of the study, however was that there was no experimental evidence that EGFR functioned

in entry *per se* nor was it determined whether EGFR was required for the delivery of virion components across the plasma membrane. Also, conflicting results exist in the literature. Fairley and colleagues demonstrated that HCMV promoted inactivation of EGFR phosphorylation and signaling (Fairley *et al.*, 2002). In these experiments, EGFR polyclonal antibodies had no effect on HCMV entry (Soderberg *et al.*, 1993a). It is important to note that EGFR is not expressed on all HCMV susceptible cells, such as those of a hematopoeitic lineage.

Finally, an anti-idiotype antibody to viral envelope glycoprotein H (gH), identified a phosphorylated 92.5kDa cell surface glycoprotein that may be involved in the steps that follow attachment (Keay and Baldwin, 1991, 1992, 1996; Keay *et al.*, 1989, 1995). Combined, the study of HCMV entry receptors, like studies aimed at identifying entry mediators in other herpesviruses, include reports that cannot be reconciled with the ability of this virus to enter and uncoat in a broad range of cell types without invoking functional redundancy. There is continued need for data confirmation as well as further functional investigation of all receptor candidates.

Entry activated cell signaling

The first and foremost observation about HCMV biology was its namesake characteristic, cytomegaly, or cell enlargement. In vitro studies initially demonstrated a unique cytopathogenic effect (CPE) of infected cells, with HCMV infected cells appearing rounded and developing an enlarged appearance with both intracellular and intranuclear inclusion bodies late during infection (Albrecht and Weller, 1980). Infection proceeded with two waves of cell rounding, the first beginning as early as a few hours postinfection and corresponding to the impact of entry, and another starting at approximately 24 hours postinfection, when there is a distinct peak in cellular transcription and translation. The cause of this phenomenon was widely speculated upon however, and theories for HCMV-induced cell rounding included cation influx, suppression of fibronectin synthesis and integrin down-regulation (Albrecht *et al.*, 1983; Albrecht and Weller, 1980; Ihara *et al.*, 1982; Warren *et al.*, 1994).

It has been apparent for many years that cells respond to HCMV virions by activation of numerous cell signaling pathways including changes in Ca^{2+} homeostasis, activation of phospholipases C and A2, as well as increased release of arachidonic acid and its metabolites (for review, see Fortunato *et al.*, 2000). All of these changes can be triggered by UV-inactivated virions (Boldogh *et al.*, 1990, 1991b), suggesting that structural components of the virus are responsible for activation during virus-cell contact and/or virus entry. Virus–cell contact also results in the activation of transcription factors such as cfos/jun, myc, NF-κB, SP-1, as well as phosphatidylinositol 3-kinase (PI3-kinase) and mitogen-activated protein (MAP) kinases ERK1/2 and p38 (Boyle *et al.*, 1999; Kowalik *et al.*, 1993; Yurochko *et al.*, 1995, 1997; Boldogh *et al.*, 1991a; Johnson *et al.*, 2001)). These virally induced cellular physiological changes are associated with a profound effect on host cell gene expression. The levels of hundreds of host cell transcripts are altered within a few hours after exposure to virus, virus particles or soluble gB (Browne *et al.*, 2001; Simmen *et al.*, 2001; Zhu *et al.*, 1998). Thus, transcriptional changes immediately after infection do not reflect viral gene expression. These data are consistent with the interpretation that HCMV engages a cellular receptor(s) that activate signal transduction pathways culminating in reprogramming of cellular transcription.

Cellular integrins may serve as coreceptors for betaherpesviruses

Cellular integrins are ubiquitously expressed cell surface receptors that, when activated, lead to major reorganization of the cytoskeleton. Integrins exist on the plasma membrane as non-covalently linked heterodimers consisting of an α- and a β-subunit, which convey specificity in cell–cell and cell–ECM (extracellular matrix) attachment, immune cell recruitment, extravasation, and signaling (Berman and Kozlova, 2000; Berman *et al.*, 2003; Cary *et al.*, 1999). In addition, integrins have emerged as receptors for a broad range of pathogens and mediate binding of plant spores, bacteria and viruses. Feire *et al.* (2004) investigated the role that integrins play in the HCMV entry pathway. Analysis of the effects of various neutralizing antibodies implicated α2β1, α6β1, and αVβ3 integrins in entry (Feire *et al.*, 2004). Furthermore, cells devoid of β1 integrin exhibited dramatically reduced susceptibility to infection with HCMV or MCMV while entry and spread were restored when the expression of β1 integrin was re-introduced into cells. Integrin-blocking antibodies did not prevent virus attachment but specifically inhibited the delivery of a virion component, pp65, into cells suggesting that integrins function at a post-attachment stage of infection, possibly at the level of membrane fusion. The involvement of multiple integrin heterodimers is consistent with integrin biology in that many natural integrin ligands, such as extracellular matrix proteins, bind to a variety of heterodimers. Furthermore, other integrin-binding viruses are characterizd

by binding to a number of different integrin heterodimers (Stewart *et al.*, 2003).

Integrins are capable of engaging ligands through a number of identified ECM protein motifs, the most common of which contain the amino acid sequence RGD. However, there are a number of RGD-independent integrin binding motifs, including the disintegrin domain proteins of the A Disintegrin and A Metalloprotease (ADAM) family of proteins. After inspection of all HCMV structural glycoproteins, the strongest homology to an integrin-binding domain was a disintegrin-like consensus sequence (RX_{5-7} DLXXF/L) (Eto *et al.*, 2002; Stone *et al.*, 1999; Wolfsberg *et al.*, 1995) on the amino-terminus of gB. Sequence alignments confirmed that the gB disintegrin loop was more than 98% identical among 44 clinical isolates analyzed. The role of this sequence in entry was confirmed through the use of synthetic peptides that inhibited both HCMV and MCMV entry, but had no impact on entry of HSV, correlating with the lack of a disintegrin-loop in gB of this virus. Furthermore, the HCMV gB disintegrin-loop was conserved throughout much of the gamma and all of the betaherpesvirus subfamilies, but not in the alphaherpesvirus subfamily where previously identified RGD sequences appear to carry out interactions with integrins. The presence of integrin-binding sequences among conserved herpesvirus glycoproteins strongly suggests that integrins may be important for entry and signaling throughout this medically important family. EGFR has also been shown to become phosphorylated and signal indirectly, as a result of integrin activation through src family kinases or focal adhesion kinase (FAK) (Jones *et al.*, 1997; Miyamoto *et al.*, 1996; Moro *et al.*, 1998). Future work will no doubt be aimed at an analysis of the integrin-triggered signaling events and defining their roles in entry and infection.

Activation of innate immunity during entry

The cellular response to HCMV particles includes dramatic upregulation of interferon stimulated genes, including interferon β itself and inflammatory cytokines; indicators of host innate immunity (Browne *et al.*, 2001; Simmen *et al.*, 2001; Zhu *et al.*, 1998; Yurochko and Huang, 1999). Toll-like receptors (TLRs) are ancient, conserved, pathogen sensors now well appreciated to activate signal transduction pathways that lead to induction of antimicrobial/antiviral genes and inflammatory cytokines (Akira, 2001). Until recently, however, TLRs were not known to recognize virus particles. To date, members of the herpesvirus, retrovirus and paramyxovirus families have been shown to be subject to innate sensing by TLRs (Compton *et al.*, 2003; Haynes *et al.*, 2001; Kurt-Jones *et al.*, 2000; Rassa *et al.*, 2002). In particular, TLR2 on PBMCs recognize HCMV particles or virus in a comparable manner, suggesting that binding and/or entry events involve the activation of this receptor. Soluble gB is able to induce a similar pattern of innate immune gene expression and can induce an antiviral state in cells (Boehme *et al.*, 2004; Boyle, Pietropaolo and Compton, 1999). Another envelope glycoprotein (gH) activates cells (Yurochko *et al.*, 1997) and the gH/gl/gO complex may contribute to a pattern of innate immune activation as a component of entry (Netterwald *et al.*, 2004), Guerrero M. and T. Compton, unpublished observations). TLR2 stimulation results in activation of NF-κB and stimulation of inflammatory cytokine production (Compton *et al.*, 2003). A common theme has emerged implicating viral envelope glycoprotein as a specific molecular trigger for TLR activation and that viral gene expression is not required (Boehme and Compton, 2004). These studies suggest a heretofore-unknown host cell response that detects viruses during entry, prior to the onset of replication events and products such as double stranded RNA that have long been recognized as TLR ligands (Boehme and Compton, 2004). Innate sensing of viruses during entry does not result in signaling that is essential to viral replication and appears more important as a determinant of the host cell response. The interaction of envelope glycoprotein and TLR suggests that entry and innate sensing may be coordinated in some manner.

Roles of betaherpesvirus envelope glycoproteins in virus entry

The HCMV envelope is exceedingly complex and currently incompletely defined. The HCMV genome encodes ORFs to at least 57 putative glycoproteins; far more than other herpesviruses, however, the extent of transcription, translation and function of the majority of these glycoproteins remains unknown. Biochemical studies of HCMV virions have revealed that 14 structural glycoproteins; eight of which have been experimentally shown to reside in the envelope (Britt *et al.*, 2004). HCMV appears to rely on herpesvirus-common homologues for entry. These include herpesvirus-common gene products gB and gH as well as glycoproteins L (gL), O (gO), M (gM), and N (gN). A number of other structural glycoproteins (gpTRL10, gpTRL11, gpTRL12 and gpUL132, gpUS28) are HCMV-specific (Table 16.1) but so far have no role in entry.

For years, the large genome and complicated reverse genetics system have made the creation of HCMV knockout and mutant viruses difficult. Recently, a system capable of such mutations was developed whereby HCMV is

Table 16.1. Envelope proteins of CMV

ORF	Protein name	Essential	Complex partner	Role in entry
UL4	gpUL4; gp48	No	None known	None known
UL33	UL33	No	None known	None known
UL55	gB	Yes	None known	Receptor binding, fusion, Signal transduction Innate immune activation
UL73	gN	Yes	UL100; gM	None known
UL74	gO	Moderate defect in cell to cell spread	UL75;gH UL115; gL	Enhancer of cell–cell spread
UL75	gH	Yes	UL74;gO UL115;gL	Fusion, receptor binding (?)Innate immune activation
UL100	gM	Yes	UL73;gN	HSPG binding
UL115	gL	Yes	UL75;gH UL74;gO	Required for gH activity
TRL10	gpTRL10	Not determined	None known	None known
TLR12	gpTRL12	Not determined	None known	None known
US27	gpUS27	No	None known	None known
US28	gpUS28	No	None known	None known

maintained as an infectious bacterial artificial chromosome (BAC) within *Escherichia coli* (Borst *et al.*, 1999). This development has greatly hastened the process of mutating individual ORFs and will generate much information regarding both the structure and function of many envelope glycoproteins. In fact, the BAC system has demonstrated the requirement for several glycoprotein genes in the production of replication competent virus (Dunn *et al.*, 2003; Hobom *et al.*, 2000; Yu *et al.*, 2002). The HCMV glycoprotein homologues gB, gM, gN, gH, gL, have been shown to be essential for growth, while gO knockout virus remained viable with a small plaque phenotype (Hobom *et al.*, 2000). Genes for all the currently identified HCMV-specific envelope glycoproteins, including UL4 (gp48), TRL10 (gpTRL10), TRL11 (gpTRL11), TRL12 (gpTRL12), US27, UL33, UL132, have been shown to be dispensable for replication and therefore are not critical for entry (Dunn *et al.*, 2003). The HCMV-encoded chemokine receptor US28 is present in the virion envelope and has been shown to promote cell–cell fusion mediated by HIV and VSV viral proteins, however the gene has been shown to be non-essential and there is no evidence for a role for gpUS28 in either HCMV–cell or cell–cell fusion events (Dunn *et al.*, 2003; Pleskoff *et al.*, 1997, 1998).

Essential and abundant HCMV envelope glycoproteins conserved throughout the herpesviruses (including gB and gH:gL) were classified as distinct disulfide-linked high molecular weight complexes (gCI and gCIII) in HCMV-infected cells (Gretch *et al.*, 1988). The gCI complex is composed of homodimers of gB (Britt, 1984; Britt and Auger, 1986) and the gCIII complex is a heterotrimeric complex composed of gH, gL, and gO (Huber and Compton, 1997, 1998; Li *et al.*, 1997). The designation gCII has been applied to a heterodimeric complex composed of gM and gN (Mach *et al.*, 2000).

At least two glycoprotein complexes have heparan sulfate proteoglycan (HSPG) binding ability, gB and the gM component of the gM:gN complex, suggesting a critical role for cell surface proteoglycan in initial virus:cell contact (Carlson *et al.*, 1997; Kari and Gehrz, 1993). Heparin binding is a property that HCMV shares with other herpesviruses, and gB is the common glycoprotein involved in this activity. HCMV gB also appears to be the primary receptor binding protein. Soluble forms of gB exhibit biphasic binding properties and cells treated with gB are refractory to infection suggesting that gB ties up critical receptor sites used by the virus (Boyle and Compton, 1998). One of the binding sites for gB is HSPGs in that cells lacking HSPGs had a single component Scatchard plot as compared to a biphasic plot for HSPG bearing cells. As noted above, it now seems clear that a second binding partner is an integrin (Feire *et al.*, 2004) but much work remains to formally prove the disintegrin hypothesis and confirm the role of this domain in receptor engagement. The gB protein may also engage EGFR (Wang *et al.*, 2003), at least in certain cell types, however it is not yet known if this interaction requires initial interaction with integrin. The gH complex may also have a distinct receptor. Syngeneic monoclonal anti-idiotypic antibodies were created that bear the "image" of this glycoprotein complex (Keay *et al.*, 1988) and led to a putative gH receptor (Keay and Baldwin, 1989, 1991, 1992, 1996). These investigations relied heavily on

a single reagent (anti-idiotypic antibodies) and has led to only a partially sequenced receptor clone, lacking homology to known human proteins (Baldwin *et al.*, 2000; Keay and Baldwin, 1996). Thus, the identity of a HCMV gH/gL/gO receptor remains unknown. Since HCMV gH and gL are essential and infectivity can be neutralized with gH antibodies, anti-idiotypic antibodies can neutralize infectivity and the closest relative of HCMV, HHV-6, contains an analogous complex consisting of the herpesvirus-common gH and gL with the product of U100 (gQ), a complex that has been shown to interact with a candidate receptor (Santoro *et al.*, 1999). HHV-6 U47 is the homolog of HCMV UL74 (gO), and is also involved in complex formation.

Membrane fusion remains a poorly understood component of entry for any of the herpesviruses. Unlike orthomyxoviruses, paramyxoviruses, filoviruses and retroviruses that employ a single envelope glycoprotein for membrane fusion, herpesviruses appear to employ multicomponent fusion machines, with evidence that these consist of gB, gH and gL (Spear and Longnecker, 2003). Both the HCMV gB- and gH-dependent entry processes are susceptible to inhibition by neutralizing antibodies that block infection at a postattachment stage of entry, presumably at the level fusion (Bold *et al.*, 1996; Britt, 1984; Keay and Baldwin, 1991; Tugizov *et al.*, 1994; Utz *et al.*, 1989). One limitation of these conclusions, however, is the lack of a direct fusion assay and thus a role for these glycoproteins in fusion is inferred. Despite the complexity of multicomponent fusion machines, it is very likely that there are strong parallels to single component fusion proteins. Alpha helical coiled-coils critical structural domains involved in fusion that function to drive the energetic folding of membranes together. Conformational changes in fusogenic proteins bearing these coiled-coils are also a defining paradigm. Using an algorithm to detect potential structural motifs, Lopper and Compton identified heptad repeat regions in gB and gH that were predicted to form alpha helical coiled coils (Lopper and Compton, 2004). Synthetic peptides to these motifs substantially inhibit HCMV entry including virion content delivery suggesting that these motifs play a fundamental role in membrane fusion. Genetic analysis of these motifs in the context of HCMV virions will be required to further analyze the importance of alpha helical coiled coils in HCMV entry. Another fundamental question will be to determine if the gB integrin interaction is a trigger of conformational change that leads to exposure of membrane fusion domains. Intriguingly, disintegrin-bearing cellular proteins in the ADAM family are known to trigger fusion via integrin interaction in a variety of processes including sperm–egg fusion and myoblast fusion (White, 2003). Development of a reliable fusion assay is also greatly needed to begin a dissection of the biophysical properties of HCMV fusion glycoproteins.

Coordination of entry and innate immune activation

We are left with an apparent dichotomy. As HCMV enters cells to establish infection, the host recognizes the virions and activates innate immune responses. How are the two processes coordinated, or are they coordinated at all? At this time, there is no apparent role for TLRs in entry in that cell stimulation that follows this event does not contribute to replication efficiency in any observable way. It seems more likely that this is a component of the entry process where the host cell senses a pathogen-associated molecular pattern displayed on HCMV envelope glycoproteins and uses this activatation to initiate a host innate defense response. One possibility is that entry receptors such as EGFR, integrins and signaling machinery, and innate immunity sensors such as TRLs, cytoplasmic adaptors and signaling machinery, coalesce into specialized membrane microdomains with integrins playing a central ligating role. Concentration of all of these cell surface receptors into a defined platform likely facilitates cell signaling events, some of which are optimal for replication and others of which are clearly hostile to the virus. Intriguingly integrins associate with TLR2 and partition into cholesterol rich lipid rafts (Ogawa *et al.*, 2002; Triantafilou *et al.*, 2002). The complexity of events at the cell surface during the initial encounter of HCMV and cells represents an exciting opportunity to better understand the molecular underpinnings of the early virus-host interactions. The recent identification of cell surface molecules involved in the early steps in infection has greatly enhanced our knowledge of entry events in infection. Yet much remains to be done to elucidate aspects of mechanism of entry events and the corresponding innate immune activation.

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17

Immediate–early viral gene regulation and function

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Introduction

Betaherpesviruses such as human cytomegalovirus (HCMV), human herpesvirus-6A and 6B (HHV-6), and human herpesvirus-7 (HHV-7) replicate more slowly than alphaherpesviruses, are highly species-specific for infection, and establish latency in progenitor cells of the bone marrow and monocytes of the blood. HCMV has been the prototype of the betaherpesviruses for studies of gene expression and regulation. In cell culture, HCMV strains have been adapted to preferentially infect and replicate in fibroblasts. However, low passage isolates replicate well in other cell types, such as endothelial cells, macrophages and dendritic cells. In the host, HCMV replicates in macrophages, dendritic cells, colonic and retinal pigmented epithelial cells, endothelial cells, fibroblasts, smooth muscle cells, neuronal cells, glial cells, hepatocytes, and trophoblasts (Fish *et al.*, 1995, 1996; Hertel *et al.*, 2003; Ibanez *et al.*, 1991; Lathey and Spector, 1991; Maidji *et al.*, 2002; Schmidbauer *et al.*, 1989; Sinzger *et al.*, 1993, 1995, 1996). In contrast, HHV-6 and HHV-7 infect $CD4^+$ lymphocytes (Takahashi *et al.*, 1989) as well as monocyte/macrophages. Although HCMV can be transferred into and out of polymorphonuclear leukocytes via cell-to-cell contact, these cells do not permit viral replication (Grundy *et al.*, 1998; Sinclair and Sissons, 1996; Sinzger and Jahn, 1996).

Various animal betaherpesviruses have been used as models for HCMV infection. CMVs infecting seven different mammalian hosts (humans, chimpanzees, African green monkeys, rhesus macaques, guinea pigs, rats and mice) have been investigated in some level of detail. Murine CMV (MCMV) infection of mice has been the most widely used animal model. MCMV tissue tropism, virulence, latency, and reactivation exhibit similarities to those of HCMV infections (Hudson, 1979; Jordan, 1983) and many important insights have emerged from this model despite the fact that rodent and primate CMVs are evolutionarily distant relatives. Between 75 and 80 open reading frames (ORFs) of MCMV have significant sequence homology to the predicted ORFs of HCMV (Chee *et al.*, 1990; Davison *et al.*, 2003; Murphy *et al.*, 2003a,b; Rawlinson *et al.*, 1996). Rhesus CMV (RhCMV) and chimpanzee CMV (CCMV) have approximately 138 and 166 ORFs with significant homology to HCMV, respectively (Davison *et al.*, 2003; Hanson *et al.*, 2003). Current estimates suggest that HCMV has at least 165 genes, but estimates of over 190 genes have been reported, depending on the method of prediction. HCMV has 45 essential genes and 15 of them have an unknown function. The majority of the non-essential genes also have unknown functions (Dunn *et al.*, 2003). For replication in human fibroblast, 68 ORFs are completely dispensable (Dunn *et al.*, 2003). Some of the viral genes that are dispensable for replication in human fibroblast cells are required for replication in human microvascular endothelial cells or in retinal pigment epithelial cells (Dunn *et al.*, 2003). Although many of the HCMV genes are dispensable for viral growth in cell culture, studies with MCMV or RhCMV suggest that many dispensable genes are important for modulating the virus–host interaction. Approximately two-thirds of the genes of HHV-6 are collinear with the unique long (U_L) region of the HCMV genome (Chee *et al.*, 1990; Gompels *et al.*, 1995; Neipel *et al.*, 1991).

This chapter will review viral replication events, with emphasis on expression and function of the betaherpesvirus immediate early (IE) genes. Understanding betaherpesvirus replication is important for the development of better strategies to prevent and treat virus-induced disease.

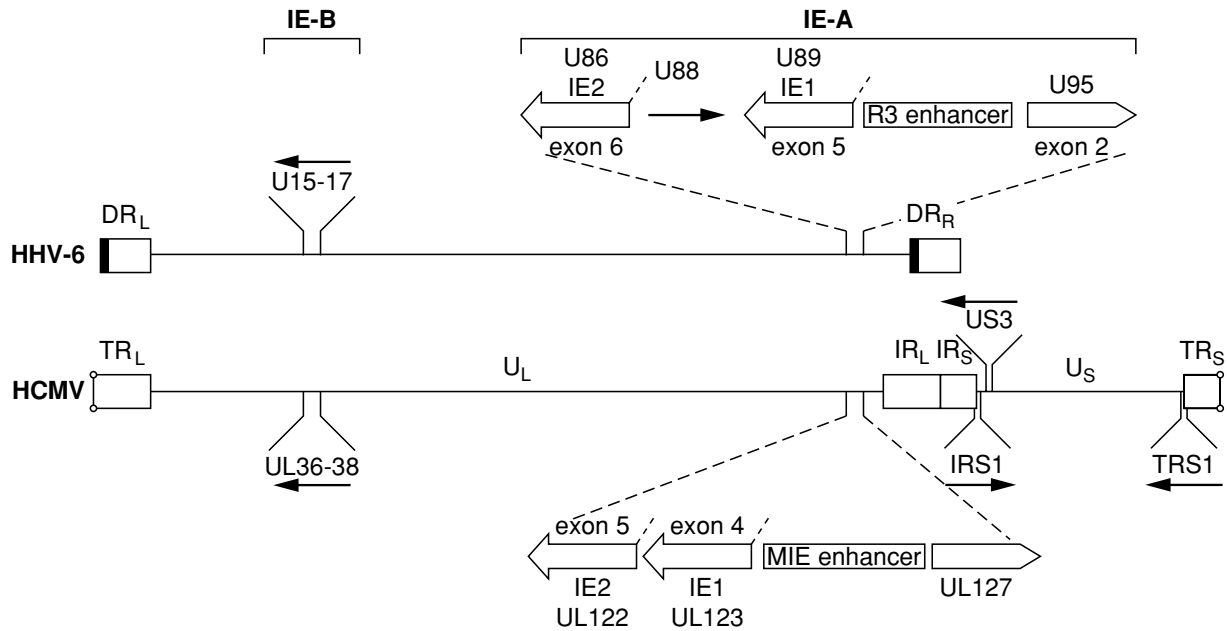

Fig. 17.1. Diagram of the immediate early (IE) genes of human cytomegalovirus (HCMV) and human herpesvirus-6 (HHV-6). The genes of the unique long (UL) components are colinear and designated alpha numerically. The unique short (US) component containing the TRS1/IRS1 and US3 genes is unique to HCMV. The unique components are flanked by either left (L) or right (R) direct repeats (DR), terminal repeats (TR), and internal repeats (IR) of the long (L) or short (S) components. Two genetically related IE loci are designated IE-A and IE-B. The major exons of the IE1 and IE2 genes in loci IE-A are designated as well as the enhancers and divergent viral gene.

Betaherpesvirus immediate early genes

The IE genes are the first viral genes transcribed after infection, and their transcription does not require *de novo* viral protein synthesis. These gene products optimize the cell for viral gene expression and replication. Figure 17.1 depicts the location of the IE gene loci on the HCMV and HHV-6 genomes. The HCMV long (L) genomic component encodes two betaherpesvirus-conserved transcription loci, the major IE genes (IE-A locus) and the IE-B locus as well as UL119-115 locus. The UL119–115 genes are not known to be involved in regulation of cellular or viral gene expression, and are not discussed in this chapter. The HCMV short (S) genomic component encodes US3 and the TRS1/IRS1 IE genes. HHV-6 lacks a region analogous to the S component of HCMV and so does not encode homologues of these HCMV genes.

The IE-A locus of HCMV encodes the major immediate early (MIE) genes and is collinear with a homologous region in HHV-6. In both viruses there is a strong transcriptional enhancer between two divergent genes (Fig. 17.1), with regulatory genes transcribed in the leftward direction designated IE1 and IE2. The function of the viral genes transcribed to the right is unknown. These viral genes and their enhancers have a major effect on productive viral replication for all betaherpesviruses where they have been studied and, consequently, they will be the focus of a majority of this chapter. For both MCMV (m128) and HCMV (UL127), the rightward transcribed viral genes in this locus are dispensable for replication in cell culture.

The IE-B locus is located in the L-component of the HCMV genome and exhibits homologous protein coding regions to HHV-6 (Flebbe-Rehwaldt *et al.*, 2000; Nicholas and Martin, 1994) (Fig. 17.1), as well as to other betaherpesviruses. The HCMV UL36–38 genes in the IE-B locus encode proteins that are required for viral growth. There are at least five transcripts from three different promoters in this region (Colberg-Poley, 1996). The products of the HCMV UL36–37 gene locus (e.g., UL37 exon 1, gpUL37, gpUL37m, and UL36) are expressed with IE kinetics and presumably serve to quickly thwart the cellular anti-viral response of apoptosis (see Chapter 21). The UL37 exon 1 unspliced RNA is abundant at IE times and remains abundant until late times after infection (Su *et al.*, 2003). HCMV oriLyt-mediated DNA replication assays are also enhanced by the UL36–38 gene products (Pari *et al.*, 1993). The HHV-6

U15–17 genes are multiply spliced in a pattern reminiscent of UL36–38 and have regulatory activities. For example, U16/17 spliced gene product activates expression from the HIV LTR promoter (Geng *et al.*, 1992).

The US3 gene is transcribed at IE times to yield three alternatively spliced transcripts (Colberg-Poley, 1996). IE gene expression is controlled at multiple levels. Upstream of the US3 promoter is two sequence repeats designated R2 and R1 (Weston, 1988). R2 is a NF-κB containing enhancer that promotes a high level of US3 transcription. R1 has 19 repetitions of a 5'-TRTCG-3' pentanucleotide arranged as direct repeats, inverted repeats, and variably spaced single pentanucleotides. Although R1 was reported to silence expression from the US3 promoter in transient transfection assays (Chan *et al.*, 1996; Thrower *et al.*, 1996), R1 enhances expression of the flanking US3 and US6 genes by an unknown mechanism when assayed in the context of viral infection (Bullock *et al.*, 2001, 2002). The US3 and US6 viral gene products disrupt the process of cellular HLA presentation of viral antigens at the cell surface and, consequently, they likely contribute to evasion of the host immune response (see Chapter 62). Between the start site of transcription and the TATA box of the US3 promoter is a *cis*-acting repressor element that binds the viral protein encoded by the essential HCMV UL34 gene (Dunn *et al.*, 2003; LaPierre and Biegalke, 2001). Mutation of the *cis*-acting element causes a high level of expression of the US3 gene at early and late times after infection (Lashmit *et al.*, 1998). The positive regulation of US3 gene expression may trap viral antigens introduced into the cell upon virus entry or expressed *de novo*. The negative regulation may prevent the toxic consequences of continued trapping of cellular HLA molecules on the membranes of the endoplasmic reticulum. US3 expression is repressed at late times after infection when the viral proteins encoded by US2, US6, and US11 contribute to immune evasion (see Chapter 62).

Because their transcription initiates in repeats flanking the U_S region, the TRS1/IRS1 genes are controlled by identical promoter elements. This arrangement results in proteins with identical N-terminal domains and divergent C-terminal domains, given the 3′-end, of their transcripts arise from different unique sequences in the U_S region (Fig. 17.1). TRS1 and IRS1 proteins are packaged into the virion as tegument components and, therefore, these viral proteins are introduced into cells in advance of IE gene expression (Romanowski *et al.*, 1997). Viruses lacking IRS1 replicate normally. However, mutation of TRS1 together with IRS diminishes the yield of infectious virus and the mutant viral particles sediment abnormally in gradients, suggesting defective viral assembly (Blankenship and Shenk, 2002). In transient assays, TRS1/IRS1 gene products activate expression of early viral promoters in cooperation with the viral MIE gene products (Romanowski and Shenk, 1997) and are necessary components for HCMV oriLyt-dependent DNA replication (Pari *et al.*, 1993). An amino terminal truncated IRS1 gene product ($pIRS1^{263}$) controlled by a downstream promoter in the unique region antagonizes the activation function of the IRS1 and TRS1 viral proteins (Romanowski and Shenk, 1997). Recent work has suggested that both TRS1 and IRS1 encode functions that evade interferon response in HCMV-infected cells (Child *et al.*, 2004). These HCMV gene products appear to be RNA-binding proteins that prevent activation of host cell protein kinase R (PKR) pathways, thereby averting shut off of protein synthesis (Hakki and Geballe, 2005). It remains possible that all of the activities previously ascribed to these IE proteins are due to this central control of the cellular response to infection as has been found for PKR inhibitors encoded by other DNA viruses.

Betaherpesvirus transcriptional enhancers upstream of the MIE genes

Transient transfection and transgenic mouse experiments with the HCMV enhancer-containing MIE promoter driving expression of an indicator gene suggest that the activity of this enhancer is influenced by the cell type and the state of cell differentiation (Baskar *et al.*, 1996a,b). However, additional transgenic mice studies yielded different data failing to show such correlation expression patterns. During infection, viral IE gene expression is first affected by attachment and entry requirements, and then, by cellular signal transduction pathways induced by the virus or other external stimuli. Serum and virion components increase MIE promoter activity at low multiplicity of infection (MOI). The relative importance of individual enhancer *cis*-acting sites may vary depending on the type of cell and external stimulus. In addition, it is not known which *cis*-acting elements in the MIE enhancer are important in a given cell type. For example, the NF-κB sites may be important in the hepatocytes under conditions of inflammation, when pro-inflammatory factors activate the transcription factor NF-κB (Prosch *et al.*, 1995). The function of the MIE enhancers of betaherpesviruses is important to understand because the enhancers likely play a pivotal role in regulating viral latency, reactivation, and pathogenesis.

Figure 17.2 compares the known consensus binding sites for eukaryotic transcription factors in enhancer elements upstream of the MIE genes of human, chimpanzee, simian, murine, and rat (England and Maastricht strains) CMVs. Uncharacterized *cis*-acting elements may emerge from

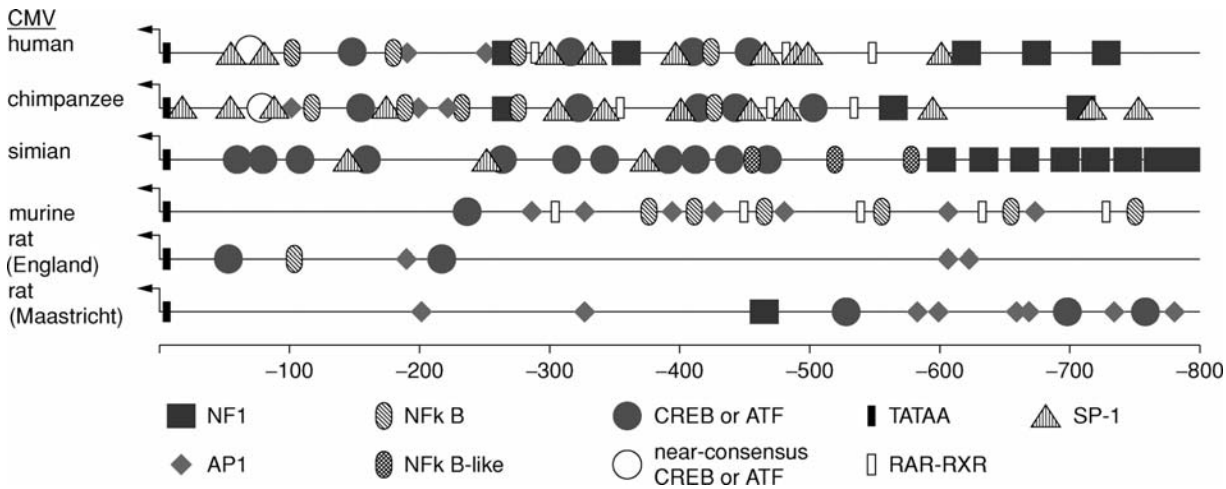

Fig. 17.2. Comparison of the enhancers of cytomegaloviruses. The known consensus binding sites of cellular transcription factors in the major immediate early enhancers are designated.

further studies seeking an impact on viral replication. CMV MIE enhancer elements have an array of *cis*-acting sites that bind cellular transcription factors (Angulo *et al.*, 1996, 1998a; Beisser *et al.*, 1998; Chang *et al.*, 1990; Davison *et al.*, 2003; Dorsch-Hasler *et al.*, 1985; Meier and Stinski, 1996; Sandford and Burns, 1996; Stinski, 1999; Thomsen *et al.*, 1984; Vink *et al.*, 2000; Meier and Stinski, 2006). Many of these binding sites are repetitive, but their arrangement and numbers vary among the different species-specific viruses. HCMV, ChCMV, SCMV, and MCMV share common regulatory elements that are functional binding sites for NF-κB, CREB/ATF, AP-1, and RAR-RXR (Angulo *et al.*, 1995, 1996, 1998a). RCMV (England strain) is unusual in that it contains fewer *cis*-acting elements than other viruses. The HCMV enhancer also has functional binding sites for serum response factor, Elk-1, Sp-1, CAAT/enhancer binding protein and gamma-interferon activating sequence (Meier and Stinski, 1996; Netterwald *et al.*, 2005). Binding sites for other cellular transcription activators are also present in this enhancer, but their significance is unknown. In transient transfection assays, the different *cis*-acting elements act individually or cooperatively to attract the RNA polymerase II transcription initiation complex to the MIE promoter (Hunninghake *et al.*, 1989).

The betaherpesvirus MIE enhancer elements are considered important because they may also serve as a target for reactivation from latency. The original hypothesis was that the MIE enhancer contributed to a quick and robust expression of the MIE genes, and evidence supporting this remains strong when expression of viruses carrying mutations in this region are studied in cell culture (Meier and Pruessner, 2000). Substitution of the enhancer of HCMV with the MCMV IE enhancer generates a virus with reduced expression and growth in culture (Isomura and Stinski, 2003), whereas subsitution of the MCMV with the HCMV MIE enhancer generates a virus that can replicate in culture or in mice (Grzimek *et al.*, 1999) with characteristics that indicated a role for the enhancer in increasing the number of cells expressing IE gene products in specific infected host tissues.

Figure 17.3 compares the enhancers of HCMV and HHV-6. Although these viruses share little common sequence, the enhancers are positioned similarly between divergent promoters upstream of the IE1 and IE2 genes (Lashmit *et al.*, 2004; Lundquist *et al.*, 1999; Takemoto *et al.*, 2001). The HHV-6 enhancer is characterized by repeat elements of 104 to 107-bp which contain polyomavirus enhancer A binding protein (PEA3) sites, NF-κB binding sites, some AP-2 binding sites (not identified in Fig. 17.3). The only obvious common elements shared by the two enhancers are NF-κB sites. The HHV-6 enhancer cannot substitute for the HCMV enhancer (H. Isomura and M. F. Stinski, unpublished data). While the HCMV enhancer influences IE1 and IE2 gene transcription, the HHV-6 enhancer has an effect on the divergent IE U95 gene transcription and is only speculated to have an effect on transcription of the IE1 and IE2 genes (Takemoto *et al.*, 2001). The HCMV MIE region has two repressor elements, one that binds IE2 proteins located immediately upstream of the IE1/IE2 transcription start site that likely represses gene expression when IE2 levels rise at early times of infection (Cherrington *et al.*, 1991; Liu *et al.*, 1991; Pizzorno and Hayward, 1990) and one that binds a cellular protein immediately upstream of the TATA box of the divergent UL127 gene and blocks expression

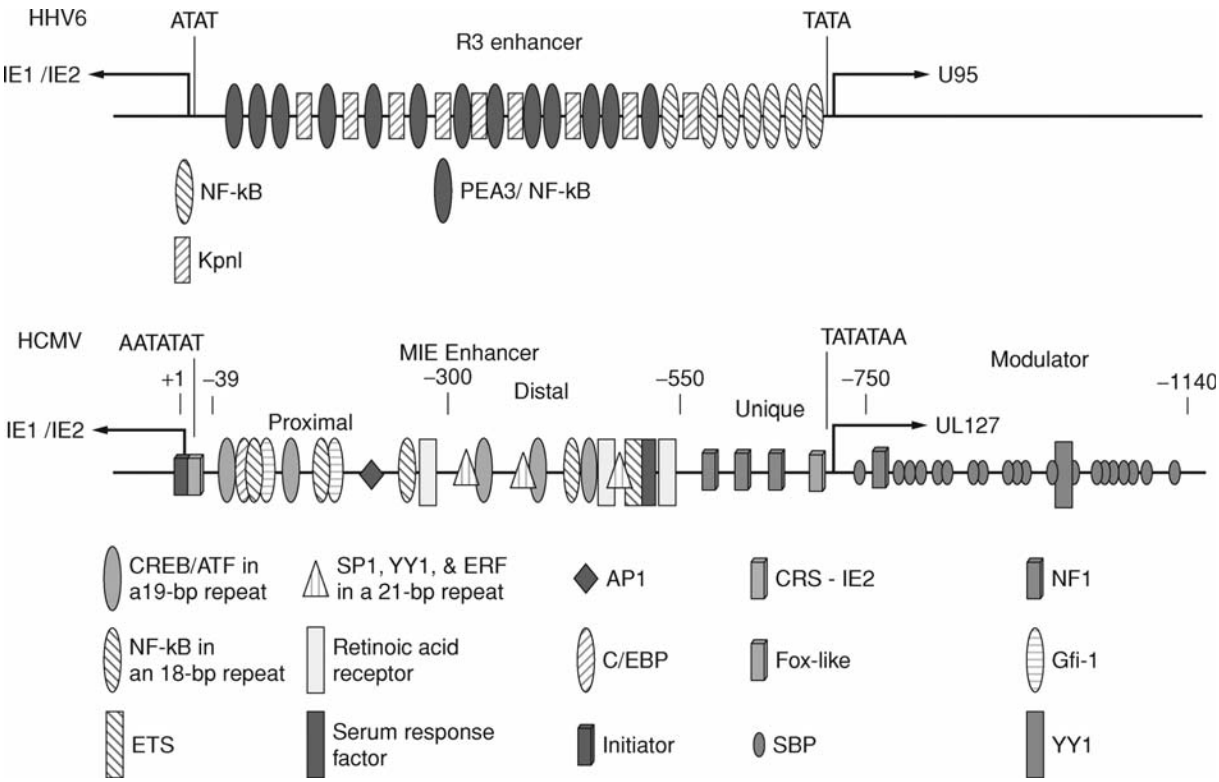

Fig. 17.3. Comparison between the HCMV major immediate early (MIE) and the HHV-6 R3 enhancer. Viral genes and promoter/transcription start sites are designated by an arrow. The HCMV also has a unique region and a modulator discussed in the text. The various transcription factor binding sites identified for HCMV and HHV-6 are designated. The AP-2 sites in the R3 enhancer are not identified. (See color plate section.)

throughout infection (Angulo *et al.*, 2000b; Lashmit *et al.*, 2004; Lundquist *et al.*, 1999). Additionally, silencing of the MIE enhancer appears to occur through a variety of cellular transcription factors in certain cell types, particularly when the cells are undifferentiated. Murine CMV has a similar arrangement of enhancer, MIE gene and divergent gene, but transcriptional repression signals have not been found. As far as has been determined, the IE1 and IE2 homologues are important in replication but the divergent genes of the betaherpesviruses are non-essential for replication in cell culture and their function is not known. The U94 gene of HHV-6, of interest because it is similar to the *rep* gene of adeno-associated virus (AAV), is located in the intron of the viral U95 gene and is transcribed in the opposite direction of U95 (Takemoto *et al.*, 2001). The AAV *rep* gene product is a DNA binding protein with ATPase and helicase activity and is required for AAV DNA replication. The HHV-6 U94 gene product is found in very low abundance during productive HHV-6 infection but shares some of these properties (see Chapter 47).

Function of the betaherpesvirus major immediate–early enhancers

Thus far, two approaches have been used to study the function of betaherpesvirus MIE enhancers during viral infection: (i) mutation of enhancer components, and (ii) substitution of the enhancer from one species with that from a different species. Mutational analysis in the context of the viral genome demonstrated that the HCMV enhancer has two functional components referred to as the distal enhancer (−580 to −300) and the proximal enhancer (−300 to −39) relative to the transcription start site (+1) (Fig. 17.3). The distal enhancer is dispensable at high MOIs in cultured cells but has a significant effect on the efficiency of viral replication at low MOIs (Meier and Pruessner, 2000). Without the distal enhancer, the virus has a small plaque phenotype. The distal enhancer is composed of multiple *cis*-acting elements. Deletion of −300 to −347 or −347 to −579 has little to no effect on MIE promoter-dependent transcription, but deletion of the entire region (−580 to −300) has

a significant effect (Meier *et al.*, 2002). The distal enhancer functions in *cis* and is orientation-independent relative to the transcription start site. The hypothetical ORFs in the distal enhancer are not important for viral replication in cell culture because insertion of stop codons at −300 or −345 had no effect on IE gene expression or virus titer (Meier *et al.*, 2002). The proximal enhancer upstream of −39 also determines the efficiency of virus replication in cell culture (Isomura *et al.*, 2004). Deletion of the proximal enhancer affects IE and early viral gene expression, viral DNA synthesis, and the rate of viral growth (Isomura *et al.*, 2004). Which elements in the distal or proximal enhancer are required for virus replication in various cell types is currently unknown. Despite the commonality of NF-κB sites in betaherpesvirus enhancers, the minimal enhancer element for HCMV appears to be an Sp-1 binding site (Isomura *et al.*, 2004, 2005).

Mutation of the CREB/ATF binding sites in the entire enhancer had little to no effect on HCMV replication in human fibroblast (HF) or in NTera2-derived neuronal cells at high or low MOIs (Keller *et al.*, 2003). Likewise, mutation of the NF-κB sites in the entire enhancer had little to no effect on viral replication at high or low MOIs in HF cells (Benedict *et al.*, 2004). Since MCMV and HCMV replicate efficiently in cells where the NF-κB activation pathway has been inhibited (Benedict *et al.*, 2004; Melnychuk *et al.*, 2003), the NF-κB transcription factor may not always be necessary for replication in cell culture. The requirement for particular HCMV MIE enhancer elements has not yet been assessed using viral mutants.

Enhancer substitution experiments have also demonstrated that CMV MIE enhancers affect viral replication. Recombinant with the rat CMV (England) MIE enhancer substituted with the MCMV MIE enhancer was deficient in replication in rat fibroblasts and in the infected rat, with greatly reduced levels of viral replication in the salivary glands (Sandford and Burns, 1996). While recombinant HCMV with the MIE enhancer substituted by the SCMV (Colburn) MIE enhancer replicated as well as wild-type virus (H. Isomura and M. F. Stinski, unpublished data), recombinant HCMV with the enhancer substituted with the MCMV enhancer replicated slower and to lower levels in HF cells (Isomura and Stinski, 2003). Consistent with this, the plaques of the enhancer substituted HCMV recombinant virus had a small plaque phenotype. When a recombinant MCMV substituted with the HCMV MIE enhancer was made, the recombinant MCMV replicated in mouse fibroblast (Angulo *et al.*, 1998b) and in mouse liver at levels similar to wild-type virus, but there was a decrease in the infection at other sites in the mouse (Grzimek *et al.*, 2001). While the MCMV enhancer is not essential for replication in cultured murine fibroblasts at high MOI, this region is required for cytopathic effects in culture and disease in the mouse (Angulo *et al.*, 1998b). Taken together, these observations suggest that CMV enhancers are not always functionally equivalent and suggest one role they play is to optimize the efficiency of viral replication in various cell types with which the virus normally interacts. The *cis*-acting elements in the betaherpesvirus enhancers have evolved over millions of years for each of the species-specific viruses.

Silencing of the immediate-early genes

Betaherpesvirus MIE genes are regulated in a cell type- and differentiation-dependent manner. The viral genomes may be organized into a nucleosome-array like latent genomes of herpesviruses in other subfamilies (Deshmane and Fraser, 1989; Dyson and Farrell, 1985). Conditionally permissive cell lines have been used to investigate silencing and reactivation of HCMV. In the undifferentiated cell, the MIE enhancer-containing promoter is silent, and the cells are non-permissive for viral replication. In the differentiated cell, the MIE enhancer-containing promoter is active, and the cells are permissive for viral replication. For example, HCMV fails to replicate after penetration into NTera2 cells, an undifferentiated embryonic carcinoma line (Gonczol *et al.*, 1984). This postentry block corresponds to silencing of the MIE promoter-dependent transcription (LaFemina and Hayward, 1986; Meier, 2001; Nelson and Groudine, 1986). Inactivity of the MIE promoter appears to be a feature of natural HCMV latency (Taylor-Wiedeman *et al.*, 1994; Kondo *et al.*, 1994, 1996; Slobedman and Mocarski, 1999). The MIE promoter of MCMV is generally inactive during viral latency, except in a rare subset of cells where spontaneous reactivation appears to be occurring (Grzimek *et al.*, 2001; Hummel *et al.*, 2001; Koffron *et al.*, 1998; Kurz *et al.*, 1999; Kurz and Reddehase, 1999).

Transient transfection studies identified the HCMV 21-bp-repeats, the unique region, and the modulator as *cis*-acting sites that confer repression of transcription in the undifferentiated monocytic THP-1 and embryonal NTera2 cell lines (Fig. 17.3) (Huang *et al.*, 1996; Kothari *et al.*, 1991; Liu *et al.*, 1994; Nelson *et al.*, 1987; Shelbourn *et al.*, 1989; Sinclair *et al.*, 1992). Three copies of the 21-bp-repeats are located in the distal MIE enhancer, whereas the unique region and modulator forms the 5′-extent of the MIE regulatory region. The following cellular repressors of transcription have also been proposed to act through one or more elements in the modulator, the unique region, or the enhancer: silencing binding protein (SBP) (Thrower *et al.*, 1996), modulator recognition factor (Huang *et al.*, 1996),

PDX1 (Chao *et al.*, 2004), Yin Yang-1 (YY1) (Liu *et al.*, 1994), methylated DNA-binding protein (Zhang *et al.*, 1995), growth factor independence-1 (Gfi-1) (Zweidler-McKay *et al.*, 1996), and the ETS2-repressor factor (ERF) (Wright *et al.*, 2002). While eliminating any one of these sets of negative *cis*-acting elements increases the MIE promoter activity in transient transfection experiments, their selective removal from the HCMV genome results in a completely different outcome. Silencing in the context of the viral genome is not alleviated by removal of the 21-bp-repeats, the modulator, the unique region, or both 21-bp-repeats and modulator in either undifferentiated monocytic THP-1 or embryonal NTera2 cells (Meier, 2001). Site-specific mutation of the Gfi-1 sites has no effect in undifferentiated monocytic THP-1 cells (R. Schnetzer and M. F. Stinski, unpublished data). Thus, silencing occurs in the context of viral infection, and its regulatory mechanism differs quantitatively from that observed in transfected cells. It remains possible that redundancy of negative *cis*-acting elements explains these differences, but studies have not yet provided any evidence of this. Nonetheless, the findings suggest that the HCMV MIE promoter becomes silenced in undifferentiated cells, but this process depends on factors that remain to be identified.

In the embryonal NTera2 cell culture model, a portion of quiescent HCMV genomes have a super-coiled structure (Meier, 2001), which appears similar to CMV latency in blood monocytes of healthy subjects (Bolovan-Fritts *et al.*, 1999). The super-coiled structure would be expected to package into nucleosomes as is the case for gammaherpesviruses. The HCMV MIE enhancer of the quiescent viral genomes is inactive even when positive-acting transcription factors NF-κB and RAR-RXR are activated (Meier, 2001). However, inhibition of cellular histone deacetylase (HDAC) reactivates transcription from the MIE promoter. These data suggest that betaherpesvirus MIE promoter silencing may involve HDAC-based modification of viral chromatin. Murphy *et al.* (2002) showed that the silent HCMV MIE promoter is associated with less acetylated histone H4 as compared to an active MIE promoter. Acetylated H4 was also less abundant on silent MIE promoters in experimentally infected blood monocytes compared to active MIE promoters in permissive monocyte-derived macrophages (Murphy *et al.*, 2002). In addition, the cellular HP1 protein, which selectively binds methylated histone H3 at lysine 9 in cellular heterochromatin (Jenuwein and Allis, 2001), is preferentially associated with the repressed MIE promoters (Murphy *et al.*, 2002). While MCMV MIE promoter reactivation can also be achieved in latently infected murine tissue (Hummel *et al.*, 2003), direct evidence for CMV DNA silencing via chromatin components is lacking at this time. The combined findings imply that chromatin may condense on the betaherpesvirus genomes in non-permissive and undifferentiated cells to restrict MIE promoter activity.

Reactivation of the immediate–early genes

Reactivation of betaherpesviruses is observed commonly in the setting of immunosuppression, particularly where allogeneic stimulation and proinflammatory cytokines are present and stimulate monocyte differentiation (Cook *et al.*, 2002; Fietze *et al.*, 1994; Hahn *et al.*, 1998; Hummel *et al.*, 2001; Mutimer *et al.*, 1997; Soderberg-Naucler *et al.*, 1997b; Soderberg-Naucler *et al.*, 2001). Proinflammatory cytokines, such as those released during allogeneic transplantation, AIDS, sepsis, or myelosuppressive chemotherapy, induce the MIE promoter-dependent transcription.

For MCMV, the effect of MIE promoter reactivation can be dampened by a mechanism that prohibits the production of the alternatively spliced ie3 RNA (Grzimek *et al.*, 2001; Kurz *et al.*, 1999), which is the functional equivalent of the HCMV IE2 gene. HCMV MIE promoter reactivation may also be subjected to further regulation in infected monocytes, as certain stimuli only reactivate production of spliced RNA for IE1, but not IE2 (Taylor-Wiedeman *et al.*, 1994). Stimuli sufficient to induce differentiation of these cells into a monocyte-derived macrophage or dendritic cell phenotype, enables completion of the HCMV reactivation program (Soderberg-Naucler *et al.*, 1997b 2001).

The molecular mechanisms that trigger and sustain CMV reactivation are largely unknown. Transgenic mice and transient transfection experiments implicate NF-κB and CREB/ATF as important mediators in stimulus-induced MIE promoter reactivation (Hummel *et al.*, 2001; Prosch *et al.*, 1995; Stein *et al.*, 1993). TNF-α potently induces NF-κB activity and reactivates MCMV's MIE promoter in latently infected lung tissue, but is not alone sufficient to sustain the reactivation process (Hummel *et al.*, 2001). HCMV reactivation from monocytes in cell culture induced by allogeneic stimulation does not require TNF-α, but instead, depends on the combination of interferon-γ and other unidentified factors (Soderberg-Naucler *et al.*, 2001). The mechanism(s) by which these cytokines or other factors stimulate HCMV reactivation is unknown. In the embryonal NTera2 cell model, forskolin stimulation of the cyclic AMP signaling pathway and inhibition of HDAC induce HCMV MIE promoter reactivation. The reactivation is dependent on CREB/ATF-binding sites within the enhancer (M. Keller and J. Meier, unpublished data). Taken together, it appears that betaherpesvirus reactivation from

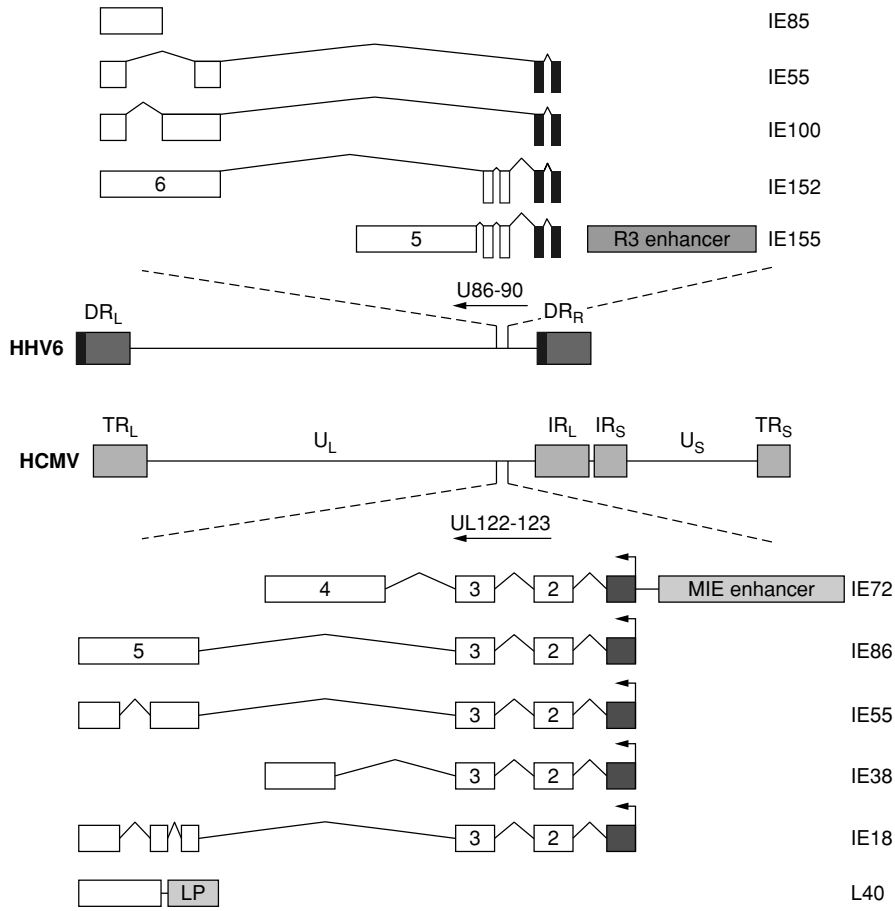

Fig. 17.4. Comparison of the IE1 and IE2 genes and their isomers for HCMV and HHV-6. The exons with ORFs for the IE1 and IE2 genes are designated as open boxes. The isomers of the IE1 and IE2 genes are designated according to apparent molecular weight. A HCMV late (L) isomer and late promoter (LP) are designated.

latency may entail multiple regulatory mechanisms for orchestrating both derepression and activation of the viral IE gene expression.

Betaherpesvirus major immediate–early genes

All betaherpesviruses have CpG dinucleotide suppression in the major immediate early (MIE) locus. The significance of CpG dinucleotide suppression is unknown, but it suggests that these genes are regulated by host methylation in a certain setting such as latency. Downstream of the betaherpesvirus enhancer-containing MIE promoter are two regulatory genes designated IE1 and IE2 in HCMV and HHV-6 as well as most other betaherpesviruses. Importantly, in MCMV, they are designated ie1 and ie3, respectively. In all betaherpesviruses, multiple gene products are encoded by these two genes through differential mRNA spicing and promoter usage throughout infection. For the CMVs, the single MIE promoter directs transcription of three small exons that are spliced alternatively to either exon 4 or exon 5 of the precursor IE RNA followed by cleavage and polyadenylation (Fig. 17.4) (Nicholas, 1994 Stenberg *et al.*, 1984, 1985). An initiation codon in exon 2 and a termination codon in either exon 4 or exon 5 gives rise to the major proteins encoded by IE1 and IE2, respectively, at IE times (Fig. 17.4). In HHV-6, the first four exons of a precursor RNA are spliced alternatively to either exon 5 or exon 6 (Fig. 17.4) (Nicholas, 1994; Schiewe *et al.*, 1994). An initiation codon in exon 3 and a termination codon in either exon 5 or exon 6 give rise to the proteins encoded by IE1 and IE2, respectively. The viral proteins encoded by HCMV are designated according to their apparent molecular weight and have amino acids in common at the amino terminus, with the exception of a late viral protein, designated L40 that arises from a unique promoter element within the exon 5

region (Fig. 17.4). HHV-6 also generates multiple forms of the IE1 and IE2 proteins by alternate splicing between and within exons. The HHV-6 MIE proteins are also designated according to their apparent molecular weight (Fig. 17.4) (Papanikolaou *et al.*, 2002).

Functions of the major immediate–early viral proteins

While it is assumed that the proteins encoded by betaherpesvirus IE1 and IE2 genes have similar functions, these proteins exhibit dramatic evolutionary divergence in amino acid sequence. These differences may have evolved to meet the regulatory needs for broadly different species-specific and cell-specific herpesviruses represented by this subgroup. The functions of the major HCMV IE1 and IE2 gene products, IE72 and IE86, have been investigated extensively, and minor products IE38, IE55, and IE18 isomers have received less attention. During the two hours after HCMV infection of HFs, the mRNA for the IE2 gene is expressed predominantly, and this is followed by a period when the mRNA for the IE1 gene predominates (Stamminger *et al.*, 1991). In contrast, the expression of HHV-6 IE1 precedes that of IE2 (Papanikolaou *et al.*, 2002). Relative to the mRNAs for HCMV IE72 and IE86 proteins, the amount of mRNA for IE55 or IE38 is low in fibroblasts. The mRNA for IE18 is lower in HFs than in macrophages (Stenberg, 1996). The IE2 protein of HCMV negatively autoregulates the expression of the IE1 and IE2 genes, but there is no evidence to date that HHV-6 IE2 protein negatively autoregulates (Cherrington *et al.*, 1991; Liu *et al.*, 1991; Pizzorno and Hayward, 1990). The IE86 protein of HCMV binds to the minor groove of the MIE promoter that contains a *cis*-repression sequence (crs) between −13 and −1 relative to the transcription start site (+1) (Lang and Stamminger, 1994). The late 40 kDa protein (L40) of the IE2 gene can also bind to the crs and negatively autoregulate transcription of the MIE genes (Plachter *et al.*, 1993; Puchtler and Stamminger, 1991; Stenberg *et al.*, 1989). While the IE1 and IE2 gene products are discussed separately below, they seem to work synergistically in executing their functions during viral infection.

The IE1 proteins

The IE1 proteins of CMVs have only a few regions of homology and a characteristic acidic acid residue cluster towards the C-termini. The IE1 genes of human and MCMV are dispensable for viral replication at high MOIs (1 to 5 PFU/cell). When the IE1 gene is deleted, the efficiency of viral replication is reduced at low MOIs (0.001 to 0.05 PFU/cell) (Greaves and Mocarski, 1998; Mocarski *et al.*, 1996). After infection at low MOI with recombinant HCMV containing an IE1 gene deletion, there are insufficient levels of early viral gene expression required for viral DNA replication (Gawn and Greaves, 2002). At high MOIs, virion-associated proteins present in infectious and non-infectious particles may compensate for the absence of functional IE1.

After synthesis in the cytoplasm, IE72 is transported to the nucleus and targeted to nuclear bodies known as promyelocytic leukemia (PML) oncogenic domains (PODs) or nuclear domain 10 (ND10). The viral protein is modified by conjugation of SUMO-1 or SUMO-2 (small ubiquitin-like modifier) at lysine residue 450. The apparent molecular weight of IE72 following modification is approximately 92 kDa (Spengler *et al.*, 2002; Xu *et al.*, 2001). Although sumoylation may be important for the efficiency of viral replication, a recombinant virus with lysine residue 450 mutated is replication competent in HFs (Lee *et al.*, 2004). Recombinant virus with lysine residue 450 mutated expresses lower levels of IE2 RNA and IE86 protein (Nevels *et al.*, 2004). With or without SUMO conjugation, IE72 displaces the ND10 presumably by binding to its associated proteins, such as PML, SP100, and hDaxx. The central hydrophobic region of IE72 binds to PML (Lee *et al.*, 2004) and inhibits the accumulation of sumoylated forms of PML. A PML-associated transcriptional repressor, HDAC-2, is inactivated (Ahn and Hayward, 1997; Ahn *et al.*, 1998a; Wilkinson *et al.*, 1998) and, consequently, the basal transcription initiation complex is activated (Muller and Dejean, 1999; Tang and Maul, 2003; Xu *et al.*, 2001). Early after infection, the viral DNA and transcripts are detected in the nucleus, and RNA polymerase and mRNA spliceosome assembly factors are juxtaposed to the ND10 (Ishov *et al.*, 1997). HHV-6 IE1 protein is also localized to ND10 and modified by SUMO-1 at lysine residue 802, but the IE1 protein does not dispense PML (Gravel *et al.*, 2002). The reason for this significant difference in the function of these betaherpesvirus IE1 proteins is not known; however, disruption of ND10 is not a requisite for HCMV replication. The multiple functions of the betaherpesvirus IE1 proteins are not fully understood. One function of the HCMV IE72 protein may be to counter the innate immune response in cells by down-regulation of virus-induced interferon-like response (Singh and Compton, 2004) and another may block apoptosis (Zhu *et al.*, 1995), promoting conditions for viral replication in the host cell.

IE38 (IE19) is reported to be an HCMV IE1 gene product that lacks amino acids 88 to 404 of IE72 (Fig. 17.4). A radioactive probe to the 5 end of IE1 detected a cDNA of 0.65 kb that could code for IE38 (Shirakata *et al.*, 2002). An antibody to a peptide between amino acids 383 and 420 detected both IE72 and IE38 (Kerry *et al.*, 1995). Others have not detected IE38 and suggest it may represent an

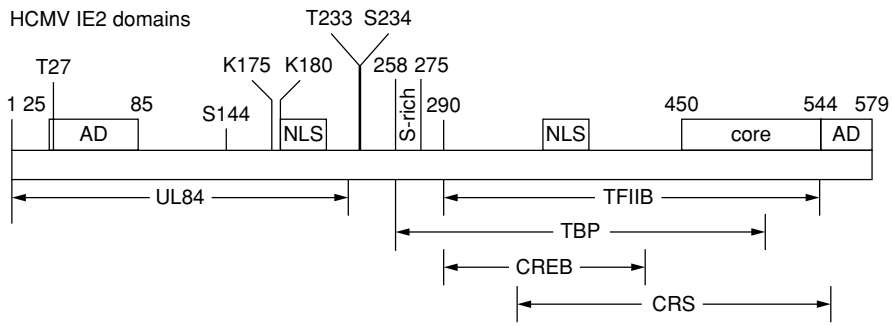

Fig. 17.5. Domains of HCMV IE86 encoded by the IE2 gene. The domains are demarcated according to amino acid residue. Transcription activation domains (AD) are located at the amino and carboxyl termini. There are serine-rich (S-rich) domains and two nuclear localization signals (NLS). There is an essential region designated the core domain. Regions of the viral protein that interact with another viral gene product (UL84), cellular transcription factors, or the *cis* repression sequence (crs) of the MIE promoter are designated.

N-terminal cleavage product of IE72 (Awasthi *et al.*, 2004). Transient transfection experiments suggested that IE38 functions synergistically with IE72 (Shirakata *et al.*, 2002). Whether or not these functions of IE38 occur in the virus-infected cell remains to be determined.

It is unclear how IE72 (or IE38) activates cellular promoters, but an association of IE72 with TATA box-associated factors (TAFs) and transcription factors (Sp-1, E2F-1, CTF-1) has been proposed (Hayhurst *et al.*, 1995; Lukac *et al.*, 1997; Margolis *et al.*, 1995). In transient transfection experiments, IE72 moderately activates the viral MIE promoter and the cellular DNA polymerase α, c-fos, c-myc and dihydrofolate reductase promoters (Cherrington and Mocarski, 1989; Hagemeier *et al.*, 1992b; Sambucetti *et al.*, 1989; Wade *et al.*, 1992). The mechanism by which IE72 protein activates promoters may be related to inhibition of HDAC-2 activity (Tang and Maul, 2003).

It has been proposed that HCMV IE72 has intrinsic protein kinase activity (Pajovic *et al.*, 1997). The related protein, IE38 does not have the proposed protein kinase domain. The Rb family members p107 and p130, the E2F transcription factor family members, and PML are phosphorylated by the IE72 protein (Ippolito *et al.*, 2003; Pajovic *et al.*, 1997). Phosphorylation of the Rb family members would dissociate the repressor from E2F and activate the E2F cellular transcription factor. The leucine zipper region of IE72 can bind the N-terminus of p107, alleviate the repressive effect of p107 and, consequently, activate cyclin dependent kinase-2 (cdk2)/cyclin E activity (Zhang *et al.*, 2003). Therefore, IE72 induces cycle cell progression, but the effect of the viral protein is more demonstrable in a p53 negative cell than in a p53 positive cell (Castillo *et al.*, 2000). An active p53 pathway should increase levels of p21 cyclin-dependent kinase inhibitor even in the presence of activated E2F transcription factors, but p21 is decreased in the HCMV-infected cell (Chen *et al.*, 2001; Noris *et al.*, 2002). HCMV infection affects p53 because the cellular protein is sequestered in the nucleus in viral replication centers (Fortunato and Spector, 1998).

The IE2 proteins

The betaherpesvirus proteins encoded by the IE2 gene exhibit amino acid similarity across their C-terminal regions. The HCMV IE2 gene and its functional homologue in MCMV (ie3) are absolutely essential for the cascade of viral gene expression (Angulo *et al.*, 2000a; Marchini *et al.*, 2001). Recombinant viruses with deletion of the HCMV IE2 gene or the MCMV ie3 gene are unable to activate early viral gene expression, and, consequently, viral DNA synthesis and late gene expression are also affected. The betaherpesvirus IE2 proteins have N- and/or C-terminal domains that activate viral gene expression and are considered a master regulator of productive infection. The IE2 gene of HCMV has been studied in detail. It regulates activation of transcription from viral and cellular promoters, negatively auto-regulates the MIE promoter, and induces cell cycle progression. Figure 17.5 is a diagram of the functional domains of the HCMV IE2 protein IE86. IE86 is also targeted to ND10 in the nucleus and modified by conjugation with SUMO-1 or SUMO-2 at lysine (K) residues 175 or 180. The apparent molecular weight of the modified protein is 105 kDa (Ahn *et al.*, 2001; Hofmann *et al.*, 2000); however, sumoylation of IE86 is not required for viral growth (Lee and Ahn, 2004).

Unlike IE72, IE86 does not disperse ND10. However, IE86 remains adjacent to ND10 where a portion of the input viral DNA is also located (Ishov *et al.*, 1997). Modification of IE86 by SUMO may facilitate interaction with the basal transcription machinery and/or with cellular or viral transcription factors. In transient transfection experiments, IE86 mutated at lysine residues 175 and 180 autoregulates the

MIE promoter, but fails to efficiently activate early viral promoters (Hofmann *et al.*, 2000). A recombinant virus with deletion of amino acid residues 136 to 290 still down-regulates transcription from the MIE promoter, but the mutant exhibited lower levels of late gene (UL83 and UL99) expression (Sanchez *et al.*, 2002). Late gene expression is also disrupted by deletion of four amino acids from 356 to 359 (White *et al.*, 2004). This mutation would disrupt a region where IE86 interacts with the basal transcription factors TFIIB and TBP (Fig. 17.5). IE86 has a serine-rich region from amino acids residues 258 to 275. Mutation of the serine residues from 258 to 264 or 266 to 269 delays viral growth, but mutation from 271 to 275 accelerates viral growth (Barrasa *et al.*, 2005). These serine residues are in a region of the protein that can bind TBP (Fig. 17.5). IE86 is also modified by phosphorylation at amino acid residues T27, S144, T233, and S234 (Harel and Alwine, 1998) (Fig. 17.5). Differences in phosphorylation may have significant effects on the function of the viral protein.

Data indicate that mutation of amino acids between 427 to 435 and 505 to 511 caused a loss of auto-regulation of the MIE promoter by IE86 (White *et al.*, 2004). Mutation of histidines 446 and 452 generates a protein that cannot bind to the crs and negatively autoregulates the MIE promoter in an in vitro transcription assay (Macias and Stinski, 1993). Therefore, the carboxyl region of the IE86 protein is critical for auto-regulation of the MIE promoter (Fig. 17.5). Autoregulation of the MIE promoter by the IE86 protein at early times is likely due to the blockage of RNA polymerase II and transcription initiation factors at the transcription start site (Macias *et al.*, 1996). At late times after infection the IE86 protein was found to be associated with repressive chromatin (Reeves *et al.*, 2006). With murine CMV, the M112/113 gene product co-localizes with and binds ie3 protein (the equivalent of human CMV IE86) to inhibit the repressive effect on the MIE promoter and promotes continued MIE gene expression (Tang *et al.*, 2005).

In transient transfection assays, multiple regions of IE86 are reported to be important for promoter activation. Two activation domains (AD) are located at amino acid 25 to 85 and 544 to 579 (Fig. 17.5). Amino acid residues 1 to 98, 169 to 194, 175 to 180, and multiple regions in the carboxyl terminal half of the protein are also important for viral promoter activation (Malone *et al.*, 1990; Pizzorno *et al.*, 1991; Sommer *et al.*, 1994; Stenberg, 1996; Yeung *et al.*, 1993). Other important regions of IE86 are amino acid residues 388 to 542, and 463 to 513, which encompass a dimerization region and a helix–loop–helix region, respectively (Ahn *et al.*, 1998b; Macias *et al.*, 1996; Macias and Stinski, 1993; Waheed *et al.*, 1998). There is a core region between amino acids 450 and 544. Mutations in this region affect most of the activities of this protein (Fig. 17.5) (Asmar *et al.*, 2004).

IE86 interacts with a wide variety of cellular transcription factors (Bryant *et al.*, 2000; Lang *et al.*, 1995; Lukac *et al.*, 1994; Yoo *et al.*, 1996). These factors include TBP, TFIIB, and TAF4 as well as histone acetyl-transferase (Bryant *et al.*, 2000; Caswell *et al.*, 1993; Fortunato and Spector, 1999; Hagemeier *et al.*, 1992a; Lukac and Alwine, 1997; Spector, 1996). IE86 may serve as a link between various upstream sequence-specific DNA binding regulators of transcription and the basal transcription initiation complex. The protein also interacts with an early viral protein encoded by the UL84 gene of HCMV (Colletti *et al.*, 2004; Samaniego *et al.*, 1994; Spector and Tevethia, 1994). Over-expression of UL84 prior to viral infection will antagonize activation of early viral promoters by IE86 protein (Gebert *et al.*, 1997). The UL84 gene is essential for viral DNA synthesis and growth (Xu *et al.*, 2004b). The UL84 protein interacts with itself and with the IE86 protein which is essential for oriLyt-dependent viral DNA synthesis (Colletti *et al.*, 2004; Sarisky and Hayward, 1996). DNA synthesis is initiated by the activation of a oriLyt bidirectional promoter by IE86 and UL84 viral proteins (Xu *et al.*, 2004a). The other HCMV IE genes, IE1, UL36–38, and IRS1/TRS1, have a stimulatory effect on ori-Lyt-mediated DNA replication in HF cells (Anders and McCue, 1996).

The IE86 protein also interacts with cellular proteins that control cell cycle progression. Several types of biological assays indicate that IE86 physically binds Rb (Fortunato *et al.*, 1997; Hagemeier *et al.*, 1994; Sommer *et al.*, 1994). The release of the cellular E2F transcription factor from Rb, as a consequence of this interaction, is considered to be one of the key HCMV mechanisms for induction of cell cycle progression. As many as 4-fold more serum-starved glioblastoma U373 or 293T cells are induced into S phase by wild type IE86 compared to a mutant protein (Murphy *et al.*, 2000). IE86 blocks cell division by arresting p53 wild type cells at G_1/S (Murphy *et al.*, 2000; Song and Stinski, 2002; Wiebusch and Hagemeier, 1999; Wiebusch *et al.*, 2003). In p53 mutant U373 cells or p53 null Saos-2 cells, IE86 does not inhibit cell cycle progression at G_1/S (Song and Stinski, 2005). These cells synthesize cellular DNA and cell cycle progression stops at the S phase for U373 cells or the G2/M-phase for Saos-2 cells (Murphy *et al.*, 2000; Song and Stinski, 2005). In p53 null Saos-2 cells, a block in cell cycle progression at the G_2/M-phase by the IE86 protein correlates with an aberrant increase in cyclin B and cdk1 levels (Song and Stinski, 2002). IE86 likely prepares the cell for DNA synthesis by activating cellular genes that regulate the cell cycle, enzymes for DNA precursor synthesis, and proteins for initiation of cellular DNA synthesis (Song and Stinski, 2002). For example, production of mRNAs for cyclin E, cdk-2, E2F-1, DNA polymerase α, and MCMs is significantly increased by IE86 (Song and Stinski, 2002). Preparation

for DNA synthesis is critical for the virus because CMVs typically infect terminally differentiated cells in the G_o/G_1 phase of the cell cycle when the pool of dideoxynucleotide triphosphates and biosynthetic enzyme levels are low. HCMV IE86 is an unusual regulatory protein in comparison to regulatory proteins of adenovirus, SV40, or papilloma DNA viruses because it pushes the p53 wild type cell from G_o/G_1 to the G_1/S transition, yet blocks further cell cycle progression (Murphy *et al.*, 2000; Wiebusch and Hagemeier, 2001). Both of the IE 72 and IE86 proteins stabilize p53, which is associated with phosphorylation of p53 at serine residue 15 (Castillo *et al.*, 2005; Song and Stinski, 2005). The IE86 protein induces the degradation of cellular mdm2 and thereby prevents ubiquitination and proteasome degradation of p53 (Zhang *et al.*, 2006). Recombinant virus with a mutant IE86 protein that fails to block cell cycle progression at the G1/S interface and allows for cellular DNA synthesis, replicates slowly relative to wild type virus (Petrik *et al.*, 2006). The IE86 protein may block rather than promote apoptosis (Zhu *et al.*, 1995).

One of the functions of cellular p53 tumor suppressor protein is to ensure termination of cells that have lost the ability to regulate growth. Even though the HCMV IE86 protein binds to p53 (Bonin and McDougall, 1997; Speir *et al.*, 1994), p53 is not inactivated by the IE86 protein and, as a result, cdk inhibitor p21 increases in relative amount (Shen *et al.*, 2004; Song and Stinski, 2002). However, in the HCMV infected cell the levels of p21 decrease (Chen *et al.*, 2001; Noris *et al.*, 2002). In the p53 wild type HF cell, IE86 induces senescence that is manifested in continued cellular metabolism, production of plasminogen activator inhibitor type I, and neutral β-galactosidase activity (Noris *et al.*, 2002).

While the viral IE86 protein acts by a different mechanism to activate cellular gene expression, it acts to favor virus survival by inhibiting cellular beta-interferon, cytokine, and pro-inflammatory chemokine expression by an unknown mechanism (Taylor and Bresnahan, 2005, 2006).

Factors that stimulate betaherpesvirus immediate–early gene expression

Cellular signal transduction events

Infection of HF cells with HCMV triggers activation of multiple signal transduction pathways. There is an activation of the phosphatidylinositol 3-kinase (PI3K), the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and the p38 MAPK signal transduction pathways (Johnson *et al.*, 2000, 2001; Rodems and Spector, 1998). Inhibitors of these signal transduction pathways suppress HCMV replication, which implies that the signal transduction pathways are necessary for efficient viral replication. The PI3K pathway is activated by virion components immediately upon entry. An activated PI3K pathway induces production of secondary messengers like phosphotidyl inositol and diacylglycerol (Albrecht *et al.*, 1990, 1991; Wang *et al.*, 2003) and increases in cyclic AMP, GMP, and intracellular stores of calcium. The MAPK/ERK and p38 MAPK pathways are activated early after infection and sustained until late times (Johnson *et al.*, 2000; Rodems and Spector, 1998). The PI3K, MAPK/ERK, and p38 MAPK pathways have major effects on the activation of a variety of cellular transcription factors. This activation often results from phosphorylation of the transcription factors and, consequently, up-regulates both cellular and viral gene expression. HCMV early promoters are suppressed by inhibitors of signal transduction pathways (Chen and Stinski, 2002). Therefore, activation of signal transduction pathways is an important step in the efficient replication of betaherpesviruses.

Prostaglandins and reactive oxygen species (ROS) also serve as secondary messengers that elicit multiple responses in betaherpesvirus-infected cells (Zhu *et al.*, 2002). Virions of HCMV up-regulate cellular cyclooxygenase-2 (cCOX-2) (Zhu *et al.*, 2002). The rhesus CMV encodes a viral COX-2 (vCOX-2), which is required for efficient viral replication in endothelial cells (Hanson *et al.*, 2003; Rue *et al.*, 2004). Prostaglandin E2, a product of COX-2, activates the HCMV MIE enhancer-containing promoter (Kline *et al.*, 1998). HCMV infection generates ROS partly through a COX-2-dependent pathway. ROS also activates the HCMV MIE promoter and augments viral replication. Inhibition of cCOX-2 or scavenging of ROS decreases HCMV IE gene expression and viral replication (Speir *et al.*, 1998; Zhu *et al.*, 2002). Treatment with prostaglandin E2 reverses the inhibitory effects on HCMV replication.

Lastly, proinflammatory cytokines induce various signaling pathways that stimulate MIE gene expression and viral replication. This outcome is observed, for example, in HCMV-infected macrophages or granulocyte–macrophage progenitors treated with tumor necrosis factor-α or interferon-γ (Soderberg-Naucler *et al.*, 1997a; Hahn *et al.*, 1998).

Virion components

A wide variety of virion components are involved in activating betaherpesvirus IE gene expression. Engagement of HCMV or viral glycoprotein gB with epidermal growth factor receptor activates the PI3K-mediated signaling pathway (Wang *et al.*, 2003). HCMV glycoproteins also have a role

in stimulating the release of pro-inflammatory cytokines mediated by the CD14 and Toll-like receptor 2 molecules on the cell surface (Compton *et al.*, 2003). These cytokines may act on signaling pathways, which in turn, activate cellular transcription factors.

HHV-6 U54 is similar in amino acid sequence to HCMV UL82. The HCMV UL82 gene and the UL35 gene, which encode viral tegument proteins, have a profound positive impact on the efficiency of viral replication (Bresnahan and Shenk, 2000; Dunn *et al.*, 2003; Schierling *et al.*, 2004). These viral tegument proteins are transported to the nucleus of the infected cell where they are targeted to the ND10 (Schierling *et al.*, 2004). UL82 (pp71) protein interacts with a cellular repressor of transcription, hDaxx, and may disrupt or inactivate the hDaxx-histone deacetylase (HDAC) complex (Cantrell and Bresnahan, 2006; Hensel *et al.*, 1996; Hofmann *et al.*, 2002; Ishov *et al.*, 1997, 2002). The viral pp71 tegument protein induces proteasome dependent degradation of hDaxx and thereby neutralizes an intrinsic immune defense mechanism of the cell (Saffert and Kalejta, 2006). In cells deficient in hDaxx, UL82 protein is not targeted to the ND10. The viral MIE promoter and early promoters are activated by UL82 protein (Baldick *et al.*, 1997; Bresnahan and Shenk, 2000; Liu and Stinski, 1992). Heterologous promoters, like herpes simplex virus IE promoters, are also activated by UL82 protein (Homer *et al.*, 1999). The mechanism involves interaction with cellular repressors of transcription and reduction of HDAC activity. The UL82 protein also has effects on cell cycle progression. It stimulates quiescent cells to re-enter the cell cycle and accelerates cells through G_1 (Kalejta and Shenk, 2003a). The UL82 protein binds to members of the retinoblastoma (Rb) protein family and induces proteosome-dependent Rb family degradation (Kalejta and Shenk, 2002, 2003b). A release of the Rb family of repressors from E2F responsive cellular promoters increases cellular cyclins, cdks, and biosynthetic enzymes for DNA synthesis. UL82 protein increases the infectivity of HCMV in HF cells (Baldick *et al.*, 1997).

HHV-6 U42 is the homologue of HCMV UL69. Deletion of the HCMV UL69 gene, which also encodes a tegument protein, causes a delay in viral DNA replication and late gene expression (Lu Hayashi *et al.*, 2000). At low MOI, virus mutated in the UL69 gene replicate slower and take longer to reach peak levels of infectious virus (Lu Hayashi *et al.*, 2000). The UL69 protein is transported to the nucleus where it may interact with proteins that regulate chromatin structure such as hSPT6 (Winkler *et al.*, 1994). The UL69 protein enhances transcription from the viral MIE promoter in transient transfection assays (Winkler *et al.*, 1994). It also affects cell cycle progression by blocking G_1/S transition (Lu Hayashi *et al.*, 2000).

Members of the betaherpesvirus family also have structural homologues of seven transmembrane G-protein-coupled receptors (GPCRs) (Chee *et al.*, 1990; Gompels *et al.*, 1995; Gruijthuijsen *et al.*, 2002; Nicholas, 1996; Rawlinson *et al.*, 1996; Vink *et al.*, 2000; Waldhoer *et al.*, 2002). Recombinant viruses with deletions of the GPCR genes replicate less efficiently in certain cell types. For example, recombinant murine or rat CMV with M33, R33, or M78 genes deleted replicate less efficiently in macrophages and are unable to disseminate to the salivary gland of the animal (Beisser *et al.*, 1999; Davis-Poynter *et al.*, 1997). Several of the viral GPCRs have been shown to initiate ligand-independent constitutive signaling through the G protein phospholipase C (PLC) pathway. Human and rhesus CMV US28 GPCRs induce intracellular signaling following binding of ligands such as fractalkine and CC chemokines. Although HCMV has a sequence homologue (UL33), US28 has been proposed to act in a manner similar to MCMV M33 in that both result in activation of the PLC pathway and activation of CREB and NF-κB. Betaherpesvirus GPCRs are postulated to have a role in the early re-programming of the host cell to favor viral replication. Their activation of cellular transcription factors may enhance IE and early gene transcription.

Infection and dysregulation of the cell cycle by betaherpesviruses

Although betaherpesviruses encode a viral DNA polymerase, processivity factor, single-stranded DNA binding protein and helicase/primase complex for viral DNA synthesis, the viruses depend on many host cell enzymes for this process. The betaherpesviruses do not encode many of the enzymes for synthesis of DNA precursors. Permissive HF cells are typically in the G_0/G_1 phase of the cell cycle and have low amounts of dideoxynucleotide triphosphates and biosynthetic enzymes of DNA precursors at the time of infection. Infection of mouse fibroblasts by MCMV or HF cells by HCMV stimulates production of cellular enzymes of DNA precursor synthesis, e.g., thymidylate synthetase, ribonucleotide reductase, deoxycytidylate deaminase, and dihydrofolate reductase (Gribaudo *et al.*, 2000; Hertel and Mocarski, 2004; Song and Stinski, 2002). Upon HCMV infection, there is a 30-fold increase in the size of the thymidylate triphosphate pool (Biron *et al.*, 1986). In addition, cells are primed for cellular DNA synthesis when the virus induces production or activities of select cellular cyclins and cdks, which are required for cell cycle progression. For example, cyclin E and cdk2, which are necessary for the G_1/S transition, are highly activated after HCMV infection of HF cells

(Bresnahan *et al.*, 1997; Jault *et al.*, 1995; McElroy *et al.*, 2000; Salvant *et al.*, 1998). In contrast, cyclin D, which is necessary for G_1 phase, and cyclin A, which is necessary for the S phase, are not induced by HCMV in HF cells (Zhu *et al.*, 1998). Betaherpesviruses appear to activate the cell differently from that of mitogenic stimulation where the cyclins are activated in a cascade fashion.

The cdks phosphorylate Rb family members and activate the E2F family of transcription factors (Nevins, 1992; Weinberg, 1995). E2F expression is increased after HCMV infection (Salvant *et al.*, 1998; Song and Stinski, 2002), which, along with cyclin E, comprises a feed-forward loop allowing amplification of signals that promote cell cycle progression from G_1 to S phase. However, with HCMV-infected HF cells, the majority of the cells are blocked at the G_1/S transition point (Bresnahan *et al.*, 1996; Dittmer and Mocarski, 1997; Lu and Shenk, 1996). Progression into S phase interferes with efficient HCMV replication because there is little to no IE gene expression during S phase (Fortunato *et al.*, 2002; Salvant *et al.*, 1998). Cellular proteins might be involved in suppressing IE gene expression during S phase, because an inhibitor of cellular proteosome activity allows for a higher level of IE gene expression (Fortunato *et al.*, 2002). In general, the G_1/S compartment of the cell cycle is the most favorable environment for betaherpesvirus gene expression, but the S phase is unfavorable.

An increase in cyclins and cdk activity is likely important for HCMV because cdk inhibitors like roscovitine and olomoucine affect splicing of the IE mRNAs (Sanchez *et al.*, 2004) and inhibit infectious virus production (Bresnahan *et al.*, 1997). In addition, inhibitors of cellular enzymes involved in DNA precursor synthesis (e.g., thymidylate synthetase and deoxycytidylate deaminase) can block HCMV and MCMV replication (Gribaudo *et al.*, 2000). The combination of inactivation of the repressor proteins such as Rb and the activation of proteins involved in movement of the cell cycle such as cyclin E and cdk2 induce an environment most favorable for betaherpesvirus DNA replication. IE proteins of betaherpesviruses play an important role in this stage of the viral replication cycle.

Summary

Betaherpesviruses infect multiple cell types in the host. In general, the productive infection ensues in cells that are terminally differentiated and in the G_o/G_1 phase of the cell cycle. Expression of the IE genes is tightly regulated because these gene products are required for viral replication and have a potential detrimental impact on viral latency. The virions contain components that stimulate both cellular and viral genes. Viral glycoproteins, tegument proteins, and GPCRs can stimulate signal transduction pathways that activate cellular transcription factors and enhance the efficiency of viral replication. The MIE gene products directly activate early viral genes and further stimulate the cell for efficient early and late viral gene expression. Both viral tegument proteins and the MIE proteins cause dysregulation of the cell cycle. The cell is pushed from the G_0/G_1 phase to the G_1/S transition point, with concomitant activation of cellular proteins for DNA precursor synthesis, cell cycle progression, and DNA initiation and synthesis. In the HCMV-infected cell, the level of phospho-serine15-p53 is increased, which stabilizes p53. In p53 wild type cells, the cell cycle stops at the G_1/S transition. The HCMV UL69 tegument protein also prevents transition into S phase. Replication of betaherpesviruses appears to be best in a cell that progresses to the G_1/S transition point, but is prevented from entering the S phase. The betaherpesviruses include both human and animal pathogens for which there are no vaccines, and the available antiviral therapies are fraught with limited efficacy and high rates of adverse effects. A better understanding of betaherpesvirus replication and pathogenesis is needed for developing novel strategies to prevent disease by these opportunists.

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18

Early viral gene expression and function

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Introduction

Viral early genes are defined by two criteria: they require prior *de novo* synthesis of viral immediate-early (IE) and cellular proteins for their transcription, and this expression is insensitive to inhibitors of viral DNA synthesis such as phosphonoformate, ganciclovir, cidofovir, and phosphonoacetate. Close inspection of the kinetics of synthesis of this class of genes reveals multiple subgroups (for review, see Fortunato and Spector, 1999). The earliest of the early gene transcripts appear and accumulate to their highest levels by 8 hours postinfection (h p.i.) (e.g., the HCMV 2.2 kb family of transcripts – UL112–113), while the latest of the early transcripts cannot be detected until just prior to the onset of viral DNA replication (e.g., the HCMV 2.7 kb major early transcript – $\beta_{2.7}$ or TRL4) and accumulate to highest levels much later during infection when viral DNA replication is allowed to proceed. (Mocarski and Courcelle, 2001) Levels of a third subgroup increase at late times (e.g., the abundant HCMV 1.2 kb RNA – TRL7) and are partially blocked by inhibitors of viral DNA synthesis. This subgroup may be further divided into genes that are referred to as early–late or leaky–late.

This review will describe the viral factors and cellular environment required for the expression of the viral early genes, the function of the early genes with respect to viral replication, and the subversion of host cellular processes and modulation of host immune responses that are associated with the expression of these genes. Selected examples of early genes will be used to illustrate different mechanisms controlling this complex class of genes. The focus is on human cytomegalovirus (HCMV) and its replication in fibroblasts, the most extensively studied betaherpesvirus. A separate section at the end of the review is devoted to human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7).

The host cell range of HCMV permissivity in vivo is broad. The major targets of infection are cell types such as epithelial and endothelial cells as well as peripheral blood leukocytes in the myelomonocytic lineage. Infection also extends to specialized parenchymal cells such as neurons and astrocytes in the brain and retina, smooth muscle cells, and hepatocytes (Sinzger and Jahn, 1996). Infection is restricted, however, when the virus is grown in tissue culture cells. Primary human fibroblasts have been used for virus isolation as well as for most studies on the cellular and molecular biology of HCMV. Fresh clinical isolates of this virus retain the ability to replicate in cultured epithelial cells and macrophages. This is not a property of laboratory propagated strains. It should be kept in mind that the rules governing early gene expression and the functional importance of the gene products might be quite different in other cell types than in fibroblasts.

Identification of HCMV early genes

The initial identification of HCMV early genes was based on studies that measured the rate of accumulation of viral transcripts. Pulse-labeled whole cell, nuclear, or cytoplasmic RNA was hybridized to cloned subgenomic fragments or viral DNA cleaved with restriction endonucleases and fractionated by gel electrophoresis (DeMarchi, 1981; DeMarchi, 1983; DeMarchi *et al.*, 1980; McDonough and Spector, 1983; Wathen and Stinski, 1982; Wathen *et al.*, 1981). In complementary experiments, the size and abundance of various RNAs were determined by hybridization of radiolabeled viral DNA or cloned subgenomic fragments to dot blots or Northern blots of RNA isolated at various times in the infection (DeMarchi, 1983; McDonough and Spector, 1983; Wathen and Stinski, 1982). Two important points emerged from these studies. First, there is no clustering of

RNA transcripts in the genome according to their temporal expression. Furthermore, some HCMV early expression appeared to be subject to post-transcriptional controls that governed either the transport of RNA from the nucleus to the cytoplasm or the stability of the RNA in the cytoplasm. These early studies provided information on the relative abundance, size, and temporal expression of about 30% of the RNAs (Chambers *et al.*, 1999; Mocarski and Courcelle, 2001) and served as the framework for more detailed analysis of individual transcription units.

More recently, additional studies have used PCR analysis, gene arrays, and large-scale mutagenesis to classify each of the predicted HCMV ORFs as IE, early, or late and to characterize these as essential or dispensable for growth in tissue culture cells (Chambers *et al.*, 1999; Dunn *et al.*, 2003; Yu *et al.*, 2003). In one study (Chambers *et al.*, 1999), infected cell RNA was hybridized to a DNA microarray carrying oligonucleotides corresponding to the majority of the strain AD169 ORFs and four ORFs present in the Towne strain. IE transcripts were isolated from cells infected in the presence of the protein synthesis inhibitor cycloheximide and harvested at 13 h p.i., while cells treated with ganciclovir for 72 h were the source of RNAs transcribed with early kinetics. Late RNA was isolated from untreated cells at 72 h p.i., and differential sensitivity to ganciclovir was the basis for further characterization of these transcripts as early–late or late. The expression of selected transcripts not previously characterized was confirmed by further hybridization analysis. In agreement with the initial studies, there was no clear correlation of kinetic class with location or polarity of the ORF in the genome, although the majority of the US region ORFs were expressed with early kinetics.

HCMV-mediated changes in the cellular environment prior to early gene expression

Examination of the viral growth cycle reveals intricate interactions between HCMV and the host cellular machinery that optimize the environment for viral replication and prevent recognition of the infected cell by the immune system. The details of these events are discussed in depth in Chapters 16, 19, 20, 21, 58, and 59. Because early gene products are important in many aspects of infection, a brief summary is provided here.

Events triggered by the binding of the virus to the host cell

The initial contact of the virus with the cell membrane triggers physiological changes and activates signaling pathways that in many ways resemble the interferon response or the second messenger-type response that occurs during regulation via hormones and growth factors (for review, see Chapter 16 and Fortunato *et al.*, 2000). For example, there is hydrolysis of phosphatidylinositol(4,5)-biphosphate, stimulation of arachidonic acid metabolism, a transient influx of calcium, upregulation of the transcription factors Sp1 and NF-κB, and activation of the mitogen activated protein kinases (ERK1/2 and pp38) and the phosphatidylinositol 3-kinase pathways (Albrecht *et al.*, 1991; Johnson *et al.* 2000, 2001a,b; Rodems and Spector, 1998). Also associated with this immediate activation of the host cell is the induction of a subset of RNAs encoding genes induced by alpha or beta interferon (IFN-α/β) in uninfected cells (Boyle *et al.*, 1999; Browne *et al.*, 2001; Navarro *et al.*, 1998; Zhu *et al.*, 1997, 1998). The HCMV-associated induction occurs in the absence of any detectable IFN-α/β or *de novo* protein synthesis and appears to require simply the exposure of the cell to virions, non-infectious enveloped particles, or dense bodies. The up-regulation of the interferon-responsive genes, however, is tempered by the viral matrix protein pp65 as well as by one or more viral gene products or cellular factors that are expressed during the first few hours of the infection (Abate *et al.*, 2004; Browne and Shenk, 2003; Browne *et al.*, 2001). It has recently been shown that the IE1 and IE2 genes encode at least two of the proteins responsible for the downregulation of this IFN-α/β-mediated signaling response. IE2 86 kDa protein blocks the production of IFN-β and several chemokines (Taylor and Bresnahan, 2005; Taylor and Bresnahan, 2006), while IE1 72 acts further downstream to block type I interferon-mediated signaling and the induction of multiple interferon-responsive genes (Paulus *et al.*, 2006).

ND10 are sites of genome deposition and IE transcription

Upon infection of cells with a variety of DNA viruses, the morphology of nuclear structures referred to variously as ND10 (nuclear domain 10), PODs (PML oncogenic domains), or PML bodies is rapidly altered (for review, see Chapter 17). While infection with adenovirus 5 (Ad5) results in these punctate structures acquiring a reticular appearance, both HCMV and herpes simplex virus type 1 (HSV-1) infections disrupt ND10 sites and disperse associated proteins. In uninfected cells, a number of proteins including the growth suppressors promyelocytic leukemia protein (PML), Sp100, HP1, and Daxx are localized to ND10 domains, and many of these are interferon inducible. As is the case for Ad5 and HSV-1, a subset of HCMV genomes are

deposited at ND10 sites immediately following infection, and it is these genomes that provide the template for early transcription (Ishov and Maul, 1996; Ishov *et al.*, 1997). Transcripts produced at these sites are consequently in close proximity to spliceosome assembly factor SC35 domains, which may further aid in rapid expression of IE genes following infection.

Once the major HCMV IE proteins, IE1 72 and IE2 86, are expressed during the infection, these first localize to ND10 sites. While the punctate pattern of IE2 86 expression persists for a longer time, between 3 and 6 h p.i. both IE1 72 and ND10-associated proteins, including PML, become completely dispersed throughout the nucleus (Ahn and Hayward, 1997; Ishov *et al.*, 1997; Kelly *et al.*, 1995; Korioth *et al.*, 1996). Further studies involving either transfection of IE1 or IE2 expression vectors or infection with a recombinant virus unable to express IE1 72 indicate that IE2 86 protein is able to localize to ND10 sites in the absence of IE1 72, but disruption does not occur (Ahn *et al.*, 1998a; Ahn and Hayward, 1997; Ishov *et al.*, 1997). IE1 72 is required for disruption of the ND10 sites, but since the IE1 deletion mutant virus replicates well under high multiplicity conditions, this event appears not to be required for the infection to proceed. Modification of IE1 by SUMO-1 is also not required for HCMV-mediated ND10 dispersal or for viral replication, although a virus with a mutation in the IE1 sumoylation site grows more slowly (Lee *et al.*, 2004; Nevels *et al.*, 2004; Xu *et al.*, 2001). The observation that IE1 abrogates sumoylation of PML and Sp100 suggests that this is at least one element of the mechanism by which ND10 associated proteins are dispersed during the infection (Muller and Dejean, 1999).

A growing body of evidence suggests that even after IE1 72 has caused dispersal of ND10 sites, these locations remain important for viral replication. Between 3 and 8 hours p.i., aggregates of cyclin dependent kinase (cdk) 9 and cdk 7 appear in the nuclei of HCMV-infected cells. Input viral genomes and IE1 and IE2 proteins are also present at some, but not all, of these sites (Tamrakar *et al.*, 2005). When cells are infected with the IE1 72 deletion mutant virus, ND10 structures are maintained and the cdk 9 aggregates can be visualized at the periphery of the PML-containing ND10 sites. In addition, the UL112–113 early gene proteins appear to colocalize with IE2 86 at the periphery of the original ND10 sites beginning about 6 h p.i., and these nucleate viral DNA replication compartments that form later during infection (Ahn *et al.*, 1999a).

Finally, a series of studies suggest that an interaction between the HCMV tegument protein pp71 and the ND10-associated Daxx is the basis of a mechanism by which early events in the viral life cycle are initiated at ND10 sites (Hofmann *et al.*, 2002; Ishov *et al.*, 2002; Marshall *et al.*, 2002). Work using recombinant viruses has shown that the Daxx-binding ability of pp71 is required for efficient HCMV replication and IE gene expression during low, but not high, multiplicity infections (Cantrell and Bresnahan, 2005; Cantrell and Bresnahan, 2006; Saffert and Kalejta, 2006). When Daxx expression is reduced, the activity of the major IE promoter increases in transient transfection assays. Knockdown of Daxx expression also alleviates the reduced IE gene expression observed in pp71 deletion mutant virus-infected cells (Cantrell and Bresnahan, 2006; Preston and Nicholl, 2006). These findings are further explained by the observation that pp71 is required for the proteasome-mediated degradation of Daxx that begins 2 h p.i. in HCMV-infected cells. This degradation is required for efficient IE gene expression and is thought to increase gene activity by eliminating Daxx-mediated histone deacetylase recruitment to promoters (Saffert and Kalejta, 2006).

Inhibition of apoptosis

Two viral IE proteins from the UL36–38 region of the genome serve to prevent the host cell from undergoing apoptosis (for review see Chapter 21). One (vMIA) is the protein product of UL37 exon 1, and the second (vICA) is encoded by UL36. vMIA, which is essential for viral replication, travels from the endoplasmic reticulum (ER) to the Golgi and finally to mitochondria. Its expression in transiently transfected HeLa cells blocks apoptosis induced by either anti-Fas antibody plus cycloheximide or by TNF-α, and in stably transfected HeLa clones, it appears to act at a stage between activation of caspase 8 and cytochrome c release into the cytoplasm (Goldmacher *et al.*, 1999; McCormick *et al.*, 2003b). Recently, it was found that vMIA sequesters the pro-apoptotic protein Bax in the mitochondria, thus suppressing mitochondrial permeabilization (Arnoult *et al.*, 2004). The half-life and localization pattern of vICA in infected cells vary depending on the strain of virus used in the infection (Patterson and Shenk, 1999). Like vMIA, vICA is involved in preventing apoptosis in infected cells, but it acts further upstream to block cleavage of procaspase 8 and its subsequent activation (Skaletskaya *et al.*, 2001). Either protein appears to be dispensable for growth in culture so long as the other is retained. Many laboratory-adapted viral strains express non-functional UL36 (Skaletskaya *et al.*, 2001). The high degree of UL36 and UL37 exon 1 conservation across the cytomegalovirus family indicates that both contribute critical functions to replication of the virus in the host organism (McCormick *et al.*, 2003a; McCormick *et al.*, 2005).

Functions of viral early genes

Many early gene products are required for successful viral replication. In libraries of HCMV BACs constructed to disrupt each unique ORF (Dunn *et al.*, 2003; Yu *et al.*, 2003) 41–45 of the ORFs examined appear essential for replication in fibroblasts; 117 are not required but when deleted, give rise to phenotypes ranging from growth like wild type to severe impairment of viral replication. Interestingly, some of these dispensable ORFs (UL24, UL64, and US29) are required for viral growth in cell types other than fibroblasts. In addition, four of the mutants with non-essential genes deleted (UL10, UL16, US16, and US19) grow significantly better than the wild type in cell types other than fibroblasts. Early gene products constitute a significant proportion of essential loci, given that 23 to 25 essential and augmenting genes were characterized as early or early–late in the study by Chambers *et al.* (1999).

Most of the viral early genes function in one of two ways. A subset of the early products required for growth in tissue culture are directly involved in viral DNA synthesis, cleavage and packaging of the viral genome, and assembly of the virus particles (see Chapters 19 and 20). A second group of genes functions to create a cellular and extracellular environment that is optimal for viral gene expression and replication, either by modulating factors involved in the control of cellular DNA synthesis or by altering the host organism's immune response to the virus.

Genes involved directly in viral replication

The majority of the proteins required for synthesis and processing of the viral DNA are expressed with early kinetics, as are many of the factors involved in the initial stages of viral particle assembly. In conjunction with some of the IE and late viral proteins, these products provide the central functions of the viral life cycle.

Initial studies on the replication of the viral DNA identified 11 loci required for origin of lytic replication (*ori*Lyt)-dependent DNA replication (Pari and Anders, 1993; Pari *et al.*, 1993) (Chapter 19). These were conducted as complementation assays in which cloned fragments of the HCMV genome were tested for their ability to support replication of a vector containing *ori*Lyt sequences. Six of the required genes are predicted to function directly in DNA replication and are homologous to factors required for herpes simplex virus type 1 (HSV-1) DNA replication. The products of these genes are pUL54, the viral DNA polymerase; ppUL44, the polymerase processivity factor; ppUL57, a single-stranded DNA binding protein; and three proteins that comprise a helicase-primase complex: pUL70, pUL102, and pUL105. Each of these genes is expressed with early or delayed early kinetics (Chambers *et al.*, 1999; Smith and Pari, 1995). Two additional early loci, UL112–113 and UL84, were identified in the complementation assays and are required together with UL122–123, IRS/TRS1, and UL36–38, three IE loci with regulatory functions that are discussed elsewhere in this chapter.

The functions of several viral early proteins have been closely examined in subsequent work. Viable viruses with point mutations in the UL54 gene, selected on the basis of reduced sensitivity to ganciclovir and cidofovir, tend to grow more slowly than parental virus (Cihlar *et al.*, 1998; Smith *et al.*, 1997). The DNA polymerase processivity factor encoded by UL44 forms a complex with the viral polymerase and binds to double-stranded DNA, thereby stabilizing interactions with the template (Ertl and Powell, 1992; Hwang *et al.*, 2000; Weiland *et al.*, 1994). Recently, residues in the C-terminus of the polymerase have been shown to be required for the ppUL44–pUL54 interaction (Loregian *et al.*, 2004; Loregian *et al.*, 2003). The function of the UL57 gene product has not been examined directly, but by analogy to its homologous HSV-1 counterpart ICP8, this protein is predicted to bind the single-stranded DNA unwound by the helicase–primase complex (Kiehl *et al.*, 2003). The UL102 and UL105 genes have been characterized (Smith *et al.*, 1996; Smith and Pari, 1995), and biochemical studies demonstrating interactions between their protein products and the product of the UL70 gene further support the idea that, as in HSV-1, these three factors function together as the HCMV helicase-primase (McMahon and Anders, 2002).

The product of the UL84 gene is essential for viral DNA synthesis and productive infection (Dunn *et al.*, 2003; Xu *et al.*, 2003, 2004; Yu *et al.*, 2003). The protein localizes to replication centers in the nuclei of infected cells (Lischka *et al.*, 2003; Xu *et al.*, 2002), interacts with IE2 86 (Spector and Tevethia, 1994), and can promote *ori*Lyt-dependent DNA replication when core replication proteins from Epstein-Barr virus are supplied (Sarisky and Hayward, 1996). Recent studies with a BAC defective for the expression of UL84 suggest that it may regulate some of the functions of IE2 86 as well as contribute to the early formation of the replication centers. Interpretation of the results with this mutant BAC, however, is complicated by the observation that the UL84 protein provided in trans does not complement viral growth (Xu *et al.*, 2004). There is also evidence that the UL112–113 proteins localize to viral replication centers early in their formation and may play a role in the recruitment of additional factors to these sites (Ahn *et al.*, 1999b; Iwayama *et al.*, 1994; Penfold and Mocarski, 1997).

UL114 is an early gene that, although not identified in these complementation assays, appears to contribute to

efficient replication of viral DNA (Courcelle *et al.*, 2001; Prichard *et al.*, 1996). The UL114-encoded uracil-DNA glycosylase is not strictly required for growth in fibroblasts, but a mutant lacking this gene is delayed in the initiation of DNA replication.

Following synthesis, viral DNA is cleaved into genome-length segments and packaged into preformed capsids (Chapter 19). Early proteins involved are introduced only briefly here. Four early–late products (major capsid protein, minor capsid protein, minor capsid binding protein, and small capsid protein) contribute to capsid formation and are the products of the UL86, UL85, UL46, and UL48.5 genes, respectively (Gibson, 1996). Capsid formation also relies on the assemblin precursor UL80.5 and the proteinase precursor UL80a. UL89 and UL56 early gene products play roles in DNA cleavage (Buerger *et al.*, 2001; Krosky *et al.*, 2003; Underwood *et al.*, 1998), and by homology to HSV-1 proteins at least four HCMV products, most expressed with early kinetics, are predicted to be involved in packaging cleaved DNA into progeny capsids. These four proteins are encoded by the HCMV UL51, UL52, UL77, and UL104 genes and are predicted to have functions including transport of the capsids to sites of DNA packaging and formation of a structure through which DNA enters the capsid. Recently, it has been shown that the TRS1 protein also may be involved in packaging at a step that occurs after the cleavage of the DNA (Adamo *et al.*, 2004).

Preparing the cell for viral DNA replication

In a cell that is permissive for the viral infection, the expression of the early genes is associated with a cascade of events that results in the stimulation of host cell genes, particularly those encoding proteins that are required for host cell DNA synthesis and proliferation. Early studies revealed a marked increase in the levels of the enzymes ornithine decarboxylase, thymidine kinase, DNA polymerase alpha, and dihydrofolate reductase following HCMV infection (Boldogh *et al.*, 1991; Estes and Huang, 1977; Hirai and Watanabe, 1976; Isom, 1979; Wade *et al.*, 1992). More recent DNA microarray analyses show that the viral infection leads to upregulation of multiple DNA synthesis and cell cycle genes at the level of transcription (Browne *et al.*, 2001). In part, this may follow HCMV-induced hyperphosphorylation of the retinoblastoma family of proteins (Jault *et al.*, 1995; McElroy *et al.*, 2000), which likely releases the inhibition that these proteins confer to the E2F/DP transcription factors that regulate the transcription of many of these same genes (Dyson, 1998). The tumor suppressor protein p53 is stabilized in HCMV-infected cells and is sequestered in viral replication centers (Fortunato and Spector, 1998; Jault *et al.*, 1995). Other proteins that are sequestered in the viral replication centers are PCNA and RPA, which are both essential for the elongation phase of host cell DNA synthesis and may play some role in viral DNA synthesis (Dittmer and Mocarski, 1997; Jault *et al.*, 1995).

HCMV also induces elevated levels of cyclin E and cyclin B and their associated kinase activities (Bresnahan *et al.*, 1996; Jault *et al.*, 1995; McElroy *et al.*, 2000; Salvant *et al.*, 1998; Sanchez *et al.*, 2003). Cyclin E transcription is induced, and this up-regulation requires the expression of viral early genes (McElroy *et al.*, 2000; Salvant *et al.*, 1998). In contrast, multiple posttranscriptional pathways are used in the activation of Cdk1/cyclin B1 complexes (Sanchez *et al.*, 2003). The accumulation of the cyclin B1 subunit is the result of increased synthesis and reduced degradation of the protein via the ubiquitin-proteasome pathway. In addition, the active catalytic subunit of the complex, Cdk1, accumulates in virus-infected cells. This is due partially to the down-regulation of the expression and activity of the Cdk1 inhibitory kinases Myt1 and Wee1 and the accumulation of the Cdc25 phosphatases that remove the inhibitory phosphates from Cdk1. Modulation of these pathways appears to require at least some early gene expression (Sanchez *et al.*, 2003).

During this early period in the infection, HCMV also inhibits selective host cell functions, presumably to ensure that viral replication is favored over that of the host. These events lead the cell to a fully "activated" state, but it is clear that the virus primes the cell for its own DNA replication at the host's expense and has sufficiently dysregulated the cell cycle and signaling pathways to ensure that cellular DNA synthesis and cell division is blocked (Bresnahan *et al.*, 1996; Dittmer and Mocarski, 1997; Jault *et al.*, 1995; Lu and Shenk, 1996; Salvant *et al.*, 1998). In contrast to the activation of cyclins E and B, the expression of cyclin A and its associated kinase activity is inhibited by infection with HCMV (Jault *et al.*, 1995). Although the failure to induce cyclin A in the virus-infected cells probably plays a role in the blockage of cellular DNA synthesis, it has recently been found that viral early gene products also affect key steps in this process prior to the requirement for cyclin A. Briefly, DNA synthesis in eukaryotic cells is precisely regulated such that genomic DNA doubles only once during each cell cycle (Diffley, 2001; Fujita, 1999; Lei and Tye, 2001). The first step involves the assembly of prereplication complexes (pre-RC) at the replication origins. The origin recognition complex (Orc), a multisubunit complex, binds to the origins of cellular DNA replication and remains bound during most of the cell cycle (Quintana and Dutta, 1999; Tatsumi *et al.*, 2000; Vashee *et al.*, 2001). Cdc6 and Cdt1 are recruited to the complex and facilitate the loading of the family of six Mcm proteins on to DNA (Maiorano *et al.*, 2000; Nishitani *et al.*, 2000, 2001; Rialland *et al.*, 2002). Cdt1 itself is regulated by

a protein called geminin that normally accumulates during S-phase and ensures that each origin is used only once. Analysis of this process has revealed that there is a delay in the expression of the Mcm proteins in infected cells. The greatest effect is observed with Mcm5, whose levels remain low until after 32 h p.i. The loading of the Mcm proteins onto the DNA pre-RC complex is also defective in the virus-infected cells and is associated with the premature accumulation of geminin (Biswas *et al.*, 2003; Wiebusch *et al.*, 2003b). Interestingly, as is the case with cyclin B, the increased levels of geminin results from decreased levels of proteasome-mediated degradation (J. W. Choi and D. H. Spector, unpublished results).

Although there is evidence from transient expression systems that the IE1 and IE2 proteins and the virion constituent proteins UL69 and UL82 contribute to the virus-mediated alteration in cell growth control (Bresnahan *et al.*, 1998; Castillo *et al.*, 2000; Kalejta *et al.*, 2003; Kalejta and Shenk, 2003; Lu and Shenk, 1999; Murphy *et al.*, 2000; Sinclair *et al.*, 2000; Song and Stinski, 2002; Wiebusch *et al.*, 2003a; Wiebusch and Hagemeier, 1999, 2001), studies in the context of viral infection show that early gene products are also required (McElroy *et al.*, 2000; Sanchez *et al.*, 2003). A challenge remains to determine which viral genes are involved and elucidate the mechanisms governing their activity. Given the large number of early genes, most of which have not yet been studied, the task is not trivial.

Modulation of host immune responses

In addition to subversion of the intracellular machinery, HCMV needs to deal early in the infection with its survival in its human host and evasion of the immune response. This topic is discussed in detail in Chapters 58 and 59, and so is only briefly summarized here (for other reviews, see (Mocarski, 2002, 2004)). Optimization of in vivo pathogenesis and viral persistence is accomplished by effects on intracellular processes, the release of soluble factors, and regulation of cellular receptors that are involved in modulation of innate, inflammatory, and adaptive immune responses. The number of viral genes that are known to play a role in these processes is still small, and all of these have proven to be dispensable for productive infection in tissue culture. The general consensus, however, is that the large block of "non-essential" early genes in the U_S region whose functions have yet to be determined are key players in viral pathogenesis.

One well-studied mechanism of viral immune evasion involves interference with MHC class I antigen presentation by at least four gene products (US2, US3, US6, and US11) (Ploegh, 1998) (Chapter 58). At early times in the infection, HCMV also disarms the interferon-mediated branch of the host's antiviral defense. The cells become refractory to IFN-α/β-mediated stimulation of MHC class I, IRF-1, MxA, and 2′–5′-oligoadenylate synthetase gene expression, transcription factor activation, and signaling (Miller *et al.*, 1999). Viral genes that have been implicated in these events include UL83 and TRS1/IRS1 (Abate *et al.*, 2004; Browne and Shenk, 2003; Child *et al.*, 2004). In addition, there is repression of IFN-γ-mediated signal transduction, and thus cells do not respond to the presence of IFN-γ by upregulating the expression of MHC class II genes (Miller *et al.*, 1998; Sedmak *et al.*, 1994). The viral US2, and possibly US3, proteins can also downregulate HLA-DRα and DMα, two proteins that are involved in MHC class II antigen presentation (Tomazin *et al.*, 1999).

Another mechanism that HCMV uses to interfere with immune surveillance is modulation of extracellular host factors (i.e., interleukins and chemokines) that are involved in inflammatory reactions and function to activate and recruit T cells, NK cells, neutrophils, and monocytes to the sites of infection. HCMV also encodes several chemokines, cytokines, and receptors that likely play a role in the inflammatory response and in the dissemination of the virus. One factor, cmvIL-10 (UL111A), is a functional homologue of IL-10 that has been proposed to downregulate macrophage and T cell responses and hence have an anti-inflammatory role (Kotenko *et al.*, 2000; Spencer *et al.*, 2002). Another is a functional alpha chemokine, vCXCL-1 (UL146), that attracts neutrophils via CXCR2 (Penfold *et al.*, 1999). HCMV also encodes four glycoproteins with homology to G-protein-coupled seven-TM receptor proteins (UL33, UL78, US27, and US28) (Chee *et al.*, 1990a). One of these (US28) serves as a specific receptor for the CX3C chemokine fractalkine and can also bind many other CC chemokines (Gao and Murphy, 1994; Kuhn *et al.*, 1995; Mizoue *et al.*, 2001). The observation that expression of US28 in vascular smooth muscle cells causes the cells to exhibit enhanced migration towards inflammatory cytokines has led to the hypothesis that US28 may not only facilitate viral dissemination, but also contribute to the progression of vascular diseases. (Streblow *et al.*, 1999).

Transactivating functions of the major IE proteins

Since viral immediate–early (IE) gene products are the primary regulators of HCMV early gene expression, it is important to introduce these proteins and their functions. This topic is covered in detail in Chapters 17, and only the salient points are discussed here. The main sites of IE transcription are the UL122–123 (major immediate early, MIE), UL36–38, US3, and IRS1/TRS1 open reading frames (Fig. 18.1).

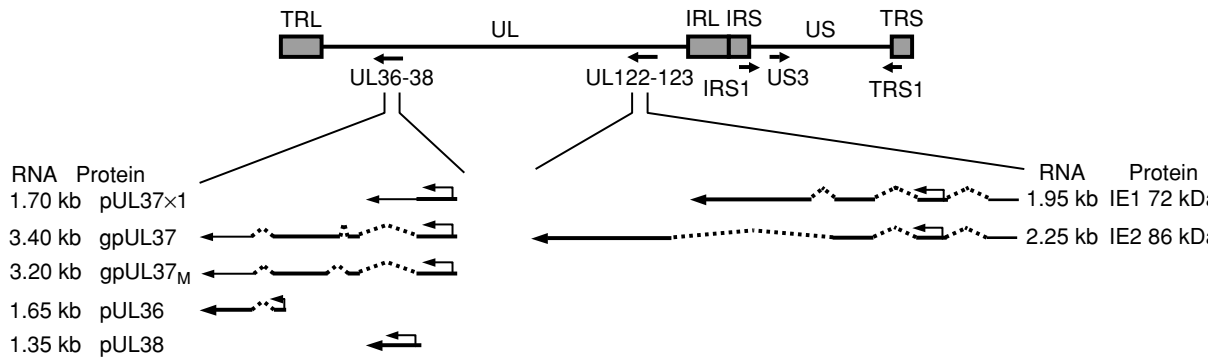

Fig. 18.1. Sites of IE transcription in the HCMV genome. Relative positions in the genome of the IE loci encoding regulatory proteins are shown. The splice patterns of the four IE transcripts expressed from the UL36–38 region and of the two major IE transcripts expressed from the UL122–123 region are indicated. Also shown is the early UL38 transcript.

The predominant and best-characterized members of this group are the products of the major IE region: the IE1 72 and IE2 86 kDa proteins and related products.

Structure and function of the IE1 72 and IE2 86 kDa proteins

The tight control and remarkably strong transactivating capacity of IE1 and IE2 proteins have caused significant effort to be directed towards understanding how they control the progression of the infection. A single, five-exon transcript from the major IE region is differentially spliced to give two predominant products the IE1 72 kDa protein (exons 1–4) and IE2 86 kDa protein (exons 1–3 and 5). Translation of each mRNA initiates in exon 2, and the two proteins share 85 amino acids (aa) at their amino termini (Stenberg *et al.*, 1984, 1985; Stinski *et al.*, 1983). IE1 72 has modest transactivating effects, including the ability to transactivate the major immediate early promoter (MIEP). Both the regions unique to IE1 and to IE2 encode additional, minor transcripts, some of which are cell-type specific (Awasthi *et al.*, 2004; Jenkins *et al.*, 1994; Kerry *et al.*, 1995; Puchtler and Stamminger, 1991; Shirakata *et al.*, 2002; Stenberg *et al.*, 1989).

IE2 86 is thought to transactivate and repress transcription via protein-protein and protein-DNA interactions. IE2 86 binds to itself, to the UL84 gene product, and to a number of cellular proteins. These host factors include components of the basal transcription complex TBP, TFIIB, and multiple TBP-associated factors (TAFs), Rb, p53, and transcription factors including Sp1, Tef-1, c-Jun, JunB, ATF-2, NF-κB, protein kinase A-phosphorylated delta CREB, p300, CBP, P/CAF, Nil-2A, CHD-1, Egr-1, and UBF (Bonin and McDougall, 1997; Bryant *et al.*, 2000; Caswell *et al.*, 1993; Chiou *et al.*, 1993; Choi *et al.*, 1995; Fortunato *et al.*, 1997; Furnari *et al.*, 1993; Gebert *et al.*, 1997; Hagemeier *et al.*, 1992, 1994; Jupp *et al.*, 1993; Lang *et al.*, 1995; Lukac *et al.*, 1994, 1997; Schwartz *et al.*, 1994, 1996; Scully *et al.*, 1995; Sommer *et al.*, 1994; Spector and Tevethia, 1994; Speir *et al.*, 1994; Wara-Aswapati *et al.*, 1999; Wu *et al.*, 1998; Yoo *et al.*, 1996) (F. Ruchti and D. H. Spector, unpublished results). While interactions between IE1 72 and cellular proteins including p107 (Johnson *et al.*, 1999) have also been demonstrated, the IE1 product does not bind to DNA. IE2 86 binds to specific DNA sequences through interactions that are thought to involve the minor groove (Lang and Stamminger, 1994; Waheed *et al.*, 1998), a notable example being its site-specific binding to the 14 bp *cis*-repression signal (*crs*) located between the TATA box and transcription start site in the major immediate–early promoter (MIEP). It has been shown that this interaction with DNA is the mechanism by which IE2 86 negatively regulates its own transcription (Cherrington *et al.*, 1991; Huang and Stinski, 1995; Lang and Stamminger, 1994; Liu *et al.*, 1991; Macias and Stinski, 1993; Pizzorno and Hayward, 1990). In addition, IE2 86 binds to similar 14 bp sites upstream of the TATA box in early promoters including the UL112–113 (2.2kb RNA), TRL7 (1.2 kb RNA), and UL4 promoters (Arlt *et al.*, 1994; Chang *et al.*, 1989; Huang and Stinski, 1995; Schwartz *et al.*, 1994; Scully *et al.*, 1995).

Multiple studies have aimed to define motifs and domains of IE2 86 that are required for both protein–protein and protein–DNA interactions as well as to identify amino acids that are likely to be post-translationally modified. The ability of IE2 86 to interact with other proteins maps broadly to the region not shared with IE1 72, amino acids 86–542 (Chiou *et al.*, 1993; Sommer *et al.*, 1994). A subset of this region, aa 388–542, is required for IE2 86 dimerization (Ahn *et al.*, 1998b; Chiou *et al.*, 1993; Furnari *et al.*, 1993). The DNA-binding capability of IE2, which controls regulation of early promoters and autoregulation, is also the result of sequences present in the C-terminal half of the

protein between residues 290–579 (Chiou *et al.*, 1993; Lang and Stamminger, 1993; Schwartz *et al.*, 1994). Regions spanning the full length of the protein appear to be important for IE2 86 transactivation of promoters, including HCMV early promoters, with the critical regions located between aa 1–98 and 170–579 (Hermiston *et al.*, 1990; Malone *et al.*, 1990; Pizzorno *et al.*, 1991; Scully *et al.*, 1995; Sommer *et al.*, 1994; Stenberg *et al.*, 1990; Yeung *et al.*, 1993). Activation of different viral promoters may require different IE2 86 domains, such as the requirement of sequences from aa 26–85 and aa 290–579 to transactivate the UL112–113 promoter and the additional requirement for aa 86–135 for activation of the 1.2 kb RNA promoter (Scully *et al.*, 1995; Sommer *et al.*, 1994).

The extensive posttranslational modifications of the major IE products suggest that they may be important for the functions of the proteins. Both IE1 72 and IE2 86 are phosphorylated and modified by sumoylation (Ahn *et al.*, 2001; Heider *et al.*, 2002a, b; Hofmann *et al.*, 2000; Spengler *et al.*, 2002; Xu *et al.*, 2001). Although a virus with a mutation in the IE1 sumoylation site grows slightly more slowly than the wild type, mutation of the IE2 sumoylation sites has no effect on viral replication (Lee and Ahn, 2004; Nevels *et al.*, 2004). In vitro and in vivo studies show that IE2 86 is phosphorylated on multiple residues (Harel and Alwine, 1998). When the consensus MAP kinase motifs at amino acids 27, 144, 233–234, and 555 are mutated to alanine, some of the resulting proteins have a stronger capacity to transactivate in transient expression assays than wild type IE2 86. However, in the context of the viral genome, these mutations have no effect on viral replication in fibroblasts (Heider *et al.*, 2002b). In contrast, mutations of the multiple serines in the region between amino acids 258 and 275 have complex effects on viral growth, with some mutations accelerating and others inhibiting the infection (Barrasa *et al.*, 2005). The fact that these major differences in growth rate are associated with only modest effects of the mutations on the transactivation function of IE2 86 in transient expression assays underscores the difficulty of extrapolating results from transient expression assays to events that occur in the context of viral infection.

In vitro and transient expression assays demonstrating the transactivating functions of the major IE proteins

Numerous studies have shown that the major IE proteins function together and separately to activate their own promoter as well as a wide range of heterologous viral and cellular promoters. Studies have been conducted primarily using transient transfection of effector plasmids expressing IE proteins and target plasmids expressing reporters driven by a range of viral promoters. In particular, these include the 1.2 and 2.7 kb RNA and UL112–113 (2.2 kb RNA) early promoters and sequences driving expression of genes involved in viral DNA replication (Colberg-Poley *et al.*, 1992; Klucher *et al.*, 1993; Schwartz *et al.*, 1994; Scully *et al.*, 1995). The results of these studies indicate that IE2 86 makes the greatest contribution to activation and in some cases, increases the level of reporter expression 40- to 80-fold over expression in the absence of IE1 72 or IE2 86. IE1 72 alone is a relatively weak transactivator, and only affects a limited number of promoters that have been tested. The transient assay function that best accounts for the growth defects exhibited by mutant viruses lacking IE1 72 is cooperation with IE2 86 in the activation of early promoters (Gawn and Greaves, 2002; Greaves and Mocarski, 1998).

Mutational analysis of the major IE products in the viral genome

The above studies laid the groundwork for elucidating the critical domains and functions of the major IE products. The recent studies that have used recombinant viruses with mutations in the UL122–123 ORFs, however, are more biologically relevant. A human fibroblast cell line expressing IE1 72 allowed the propagation of a mutant virus lacking UL123 exon 4 that was unable to express full-length IE1 72 (Mocarski *et al.*, 1996). While our laboratory and others have attempted the construction of a similar cell line expressing the IE2 86 protein, to date none has been isolated. In the absence of a complementing cell line, it is difficult to propagate recombinant viruses with mutations in essential genes like IE2 86; however, the advent of bacterial artificial chromosomes (BACs) as vectors for the cloning of herpesvirus genomes has largely allowed this problem to be circumvented (for review see Adler *et al.*, 2003). Since the majority of the viral genome is present in the BAC, mutations can be made and characterized entirely in bacteria regardless of the viability of the resulting virus. Reconstitution of virus from the clone is achieved by transfecting the altered genome and a construct expressing pp71 into cells permissive for HCMV infection (Baldick *et al.*, 1997).

Several groups have since used this approach to construct HCMV IE2 86 mutants. A recombinant virus with most of the unique region of the IE2 gene (ORF UL122) deleted is defective in early gene expression and does not produce infectious progeny, providing additional evidence that IE2 86 is required for the activation of early genes and for viral replication (Marchini *et al.*, 2001). Members of our group generated a viable mutant with a deletion spanning IE2 86 residues 136–290 and showed that this virus expresses IE and early genes and replicates its DNA

comparably to the wild type but is delayed in expression of a subset of late genes (Sanchez *et al.*, 2002).

Smaller mutations introduced into the IE2 86 gene in the viral genome have also been used to define specific regions of the protein required for the activation of early promoters. Members of our laboratory have constructed recombinants with internal deletions of amino acids 356–359, 427–435, or 505–511 (White *et al.*, 2004). These mutations were selected on the basis of the IE2 86 domain mapping and functional studies discussed above and are located in the C-terminal region important for protein–protein interactions and DNA binding. Each deletion results in a nonviable virus. The IE2 86Δ356–359 mutation removes amino acids implicated in the activation of the UL112–113 and UL54 promoters (Stenberg *et al.*, 1990) and results in a clone that is able to support limited early gene expression but not replication of viral DNA. The IE2 86Δ427–435 and IE2 86Δ505–511 mutations disrupt the zinc finger and helix-loop-helix motifs present in the protein, and the resulting recombinants do not support early gene expression. All are defective in *crs*-mediated autorepression of the major IE promoter, although the degree of this defect varies with the mutant. Similarly, a temperature-sensitive IE2 86 mutant virus that contains the point mutation C509G (C510G in AD169) is able to transactivate the UL112–113 promoter at 32.5°C, but not at 39.5°C (Heider *et al.*, 2002a). This mutant also exhibits increased transcription from immediate early loci consistent with a defect in autoregulation.

The use of other recombinant viruses has helped elucidate the contributions of IE2 86 to host cell cycle dysregulation during HCMV infection. A virus with a deletion of the majority of exon 3 of the major IE region expresses altered forms of both IE1 72 and IE2 86 proteins and is viable, but severely growth impaired (White and Spector, 2005). It is defective both in the activation of viral early promoters and in altering the expression of certain cellular proteins including cyclin E. Neither of these defects can be fully complemented by growth in the presence of wild type IE1 72 protein. C-terminal sequences of IE2 86 are also required for host cell cycle dysregulation, as infection with a virus carrying a glutamine-to-arginine point mutation at aa 548 does not arrest the host cell cycle and does allow host DNA replication to proceed (Petrik *et al.*, 2006).

Fewer HCMV mutants with disruptions in the IE1 72 coding region have been isolated and characterized, but an existing mutant constructed by deleting exon 4 of the major IE region gives important information about the role of this protein in regulation of the infection. The recombinant is viable, exhibiting minimal growth defects in cells infected at a high multiplicity but exhibits striking replication deficiency at low MOIs (Gawn and Greaves, 2002; Greaves and Mocarski, 1998; Mocarski *et al.*, 1996). Fibroblasts infected with 0.4 pfu/cell of IE1 mutant virus express IE2 86 about as frequently, and to similar levels, as wild-type infected cells, but by immunostaining, deletion mutant-infected cells express delayed early proteins, including ppUL44, ppUL57, and pUL69, much less frequently or to lower levels than wild-type infected cells. This effect on protein expression is apparent at the transcriptional level as well, with decreased accumulation of delayed early RNAs in cells infected at low multiplicity with the mutant. UL112–113 expression is supported to an intermediate degree, with fewer mutant-infected cells staining positively for these proteins than for IE2 86, but more than stained positive for ppUL44. Apparently, while high levels of IE2 86 or another factor are able to compensate for the loss of IE1 72 during a high multiplicity infection, efficient activation of early genes in cells infected at low multiplicity requires IE1 72. A further study used constructs expressing wild type or mutant forms of IE1 72 to complement the IE1 deletion mutant virus in *trans* (Reinhardt *et al.*, 2005). This work showed that aa 476–491 tether IE1 72 to chromatin but are not required for complementation, while aa 421–475 comprise an acidic domain necessary for complementation and restoration of wild type titers during low multiplicity growth.

Additional immediate early proteins have regulatory roles

In addition to UL122–123, the UL36–38 and IRS1/TRS1 families and the US3 locus encode IE proteins with regulatory functions. Knockout mutants constructed to date suggest that either UL37 exon 1 or UL36 are necessary for progression of the infection (Blankenship and Shenk, 2002; Borst *et al.*, 1999; Dunn *et al.*, 2003; Patterson and Shenk, 1999; Yu *et al.*, 2003; McCormick *et al.*, 2005). The IRS1 and TRS1 ORFs encode three proteins: two expressed from a promoter located in the repeated region flanking the US segment of the genome with an ORF continuing into the unique region, and a third, smaller, protein designated pIRS1^{263} which is expressed from an internal promoter in the unique region of the IRS1 gene (Romanowski and Shenk, 1997). In transient expression assays, either the IRS1 or TRS1 protein can complement HCMV origin-dependent DNA replication and modestly upregulate transcription from the MIEP or cooperate with IE1 72 and IE2 86 in activation of other viral promoters (Pari and Anders, 1993; Romanowski and Shenk, 1997). None of these three gene products is essential for viral replication in tissue culture, since recombinant viruses lacking the unique regions of the IRS or TRS1 open reading frames are viable (Blankenship

and Shenk, 2002; Jones and Muzithras, 1992). The mutant lacking the IRS1 products grows normally, whereas the TRS1 deletion mutant exhibits a multiplicity-dependent growth phenotype. The amount of virus released from cells infected with the TRS1 deletion mutant at a low MOI is reduced drastically compared to that released from wild-type infected cells, but cells infected at a high MOI produce only slightly less virus than the wild type. TRS1 mutant virus replication proceeds normally through DNA replication, and appears to be defective in packaging the viral DNA at a step that occurs after the cleavage of the DNA (Adamo *et al.*, 2004). Two studies have investigated TRS1 and IRS1 protein function using recombinant vaccinia or herpes simplex type 1 viruses that lack the normal ability to block host cell protein synthesis shutoff upon infection (Child *et al.*, 2004; Cassady, 2005; Hakki and Geballe, 2005). TRS1 and IRS1 proteins were able to restore this ability in both cases, either after transient expression in the vaccinia virus-infected cell or from the HSV-1 recombinant. TRS1 protein also binds dsRNA via an N-terminal domain distinct from the C-terminal region that contributes to the evasion of the shutoff of translation (Hakki and Geballe, 2005).

The UL36–38 gene products were also identified in the study that identified eleven loci required for complementation of DNA replication (Pari and Anders, 1993), and are individually dispensable for growth in culture (Dunn *et al.*, 2003; Yu *et al.*, 2003; McCormick *et al.*, 2005). Four of five transcripts from this region are expressed with IE kinetics, three from the UL37 promoter and one coding for the UL36 product (Fig. 18.1) (Chee *et al.*, 1990b; Goldmacher *et al.*, 1999; Kouzarides *et al.*, 1988). The 3.4 kb spliced transcript from the UL37 promoter is present only at IE times and encodes an integral membrane glycoprotein, gpUL37 (Kouzarides *et al.*, 1988; Tenney and Colberg-Poley, 1991a,b). An alternative splice of this transcript generates the 3.2 kb mRNA coding for gpUL37$_M$ (Goldmacher *et al.*, 1999; Colberg-Poley *et al.*, 2000), and these proteins traffic from the ER to mitochondria. While gpUL37 is able to transactivate the hsp70 promoter in transient assays, and UL37 exon 2 or exon 3 sequences are required for this activity, deletion of these exons does not affect the ability of the virus to replicate in culture (Borst *et al.*, 1999; Colberg-Poley *et al.*, 1998; Goldmacher *et al.*, 1999; Tenney *et al.*, 1993; Zhang *et al.*, 1996). The conservation of the UL37 exon 1 sequence in clinical isolates and high-passage laboratory strains, as well as in other primate CMVs, and in rodent CMVs (McCormick *et al.*, 2003b, 2005) suggest that the anti-apoptotic functions of vMIA homologues are important in response to the stress of infection. In transient transfection assays, a construct expressing UL37 exon 1 can also activate the UL54, UL44, and other early viral promoters. Although this activation is observed when constructs expressing IE1 72 and IE2 86 are used in this assay (Colberg-Poley *et al.*, 1998), adding UL37 exon 1 and IE1/IE2 expression vectors together results in a synergistic activation effect. The final IE transcript from the UL36–38 region is the UL36 RNA, which encodes a protein (vICA) that is also involved in preventing apoptosis in infected cells (Skaletskaya *et al.*, 2001). The fifth transcript from this region is the product of the UL38 gene and is expressed with early kinetics.

A third region transcribed with IE kinetics is the US3 gene, which specifies at least three alternatively spliced RNAs coding for related proteins. It is the target of complex positive and negative regulatory control (Biegalke, 1995, 1997, 1998, 1999; Chan *et al.*, 1996; Thrower *et al.*, 1996) and like much of UL36–38, is dispensable for growth in culture (Jones and Muzithras, 1992). Proteins generated from this region help the virus to evade the host immune response by keeping MHC class I molecules retained in the ER and unable to traffic to the plasma membrane (Ahn *et al.*, 1996; Jones *et al.*, 1996). US3 transcripts are abundant during the first three hours of infection and decrease by five h p.i. Transcription is regulated by a combination of elements located near the promoter including a silencer, enhancer, and transcriptional repressive element (tre) which shares sequence similarity with the *crs* element involved in control of major IE region expression (Biegalke, 1998; Bullock *et al.*, 2001, 2002; Chan *et al.*, 1996; Lashmit *et al.*, 1998; Thrower *et al.*, 1996). The product of the UL34 gene binds the tre element and represses transcription of US3 (LaPierre and Biegalke, 2001). The US3 proteins seem to possess limited intrinsic transactivating capability, as they have only been shown to induce the cellular hsp70 promoter in transient assays (Colberg-Poley *et al.*, 1992; Tenney *et al.*, 1993; Zhang *et al.*, 1996).

UL112–113 transcription is differentially controlled at early and late times

Many of our current views on the regulation of early gene expression are derived from studies on the UL112–113 region of the HCMV genome (Fig. 18.2). The UL112–113 ORFs encode a family of phosphoproteins of 84, 50, 43, and 34 kDa that share a common amino terminal domain. The initial evidence for the importance of these gene products came from the studies of Pari and Anders, who identified the locus encoding them as one of the eleven required for the replication of a plasmid containing the HCMV origin of DNA replication *ori*Lyt (Pari and Anders, 1993). As noted above, the UL112–113 proteins were found to be among

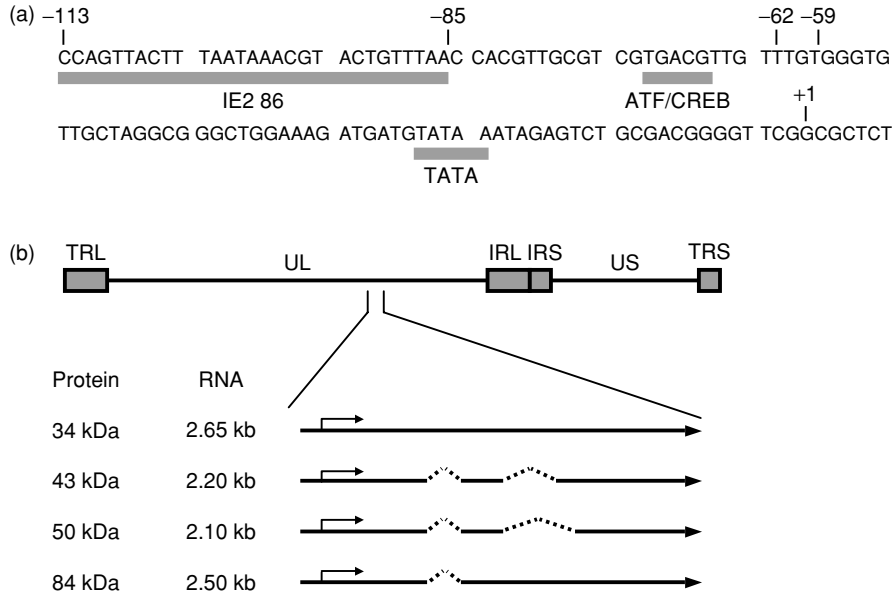

Fig. 18.2. Structure of the HCMV UL112–113 region. (a) The UL112–113 promoter from nt −113 to +7 relative to the early transcription start site, +1. The IE2 86 binding site located between −113 and −85, the consensus ATF/CREB site, and the late transcription start site at −62 are indicated. Sequences between −84 and −59 are required for IE2 86-mediated activation of the UL112–113 promoter. (b) Four transcripts from the UL112–113 region encode phosphoproteins with common N-termini. Splice patterns and sizes of the transcripts and corresponding proteins are indicated. The 2.1 and 2.2 kb RNAs are expressed by 8 h p.i., while the 2.5 and 2.65 species increase in abundance later.

the first to colocalize with IE2 86 at the periphery of the original ND10 sites and appeared to form the initial nucleation sites for subsequent viral DNA replication (Ahn *et al.*, 1999a). Transient expression assays also have shown that they can cooperate with the UL36–38 and IRS1/TRS1 ORFs to augment the stimulation of several early gene promoters by the IE1 and IE2 proteins. Although the recent studies with mutant recombinant viruses that do not express these proteins indicate that the gene products are not absolutely essential for the viral infection, the resulting viruses are severely debilitated in their ability to replicate (Dunn *et al.*, 2003; Yu *et al.*, 2003). The molecular and cellular mechanisms underlying their function, however, are still unknown.

Transcription from the UL112–113 locus begins as early as 8 h p.i. and continues for the duration of the infection, although the relative abundance of members of the family changes as the infection progresses (Staprans *et al.*, 1988; Staprans and Spector, 1986). Two spliced RNAs of 2.1 and 2.2 kb are expressed by 8 h p.i. and encode 50 kDa and 43 kDa phosphoproteins, respectively (Staprans *et al.*, 1988; Staprans and Spector, 1986; Wright and Spector, 1989; Wright *et al.*, 1988). These transcripts are coterminal at both 5′ and 3′ ends and share identical 5′ and internal exons, but use different splice acceptor sites in the 3′ exon to generate the two species, which encode proteins with different carboxyl termini. Later in the infection, transcription from the early start site decreases and initiation of transcription occurs at a site further upstream at nt −62. Two RNAs of 2.5 and 2.65 kb also increase in abundance as the infection proceeds, with the 2.5 kb transcript having spliced out only the first intron and encoding an 84 kDa phosphoprotein and the unspliced 2.65 kb transcript specifying a 34 kDa phosphoprotein (Staprans and Spector, 1986; Wright and Spector, 1989; Wright *et al.*, 1988).

In initial studies, transiently transfected UL112–113 promoter-CAT reporter constructs were used to determine that the region located at −113 to −59 relative to the transcription start site is required for activation of this promoter during the infection. These assays utilized 5′ and internal promoter deletion mutants to show that these sequences were required for activation by IE2 86; this region also contained one of four binding sites for IE2 86, at −113 to −85 (Schwartz *et al.*, 1994; Staprans *et al.*, 1988). Further work indicated that although this IE2 86 binding site contributes to full activation of the UL112–113 promoter, it is in fact sequences between −84 and −59 that are strictly required for full activation of this promoter by IE2 86 (Arlt *et al.*, 1994; Schwartz *et al.*, 1996). A consensus ATF/CREB site is located between nt −71 to −66, suggesting that a member of the ATF/CREB family of transcription factors contributes significantly to IE2 86-mediated promoter activation

(Staprans *et al.*, 1988). Additional mutational analyses of the region indicate that this contribution is modulated by interactions with factors bound to other regions of the promoter.

Further work used a series of gel shift analyses to establish that CREB is the major ATF-related protein in uninfected U373 MG cells that binds to this site (Schwartz *et al.*, 1996). Three bands were observed following incubation of wild-type UL112–113 promoter sequences from −84 to −59 with nuclear extracts from U373 MG cells, and two of these were reduced or eliminated when wild type sequences from −72 to −61 were mutated. A complex comigrating with one of the bands also formed when a DNA fragment containing a consensus ATF/CREB site was used as the probe instead of the UL112–113 promoter. The majority of this consensus probe-protein complex was supershifted by the addition of anti-CREB antibody, as was most of the corresponding band in the UL112–113 promoter-protein complexes. The other two bands were less affected by the addition of anti-CREB antibody, but also were not supershifted in the presence of either anti-ATF-2 or anti-ATF-4.

Subsequent studies from our laboratory have used recombinant viruses to define the promoter elements controlling UL112–113 expression in the infected cell (Rodems *et al.*, 1998). In these viruses, a cassette containing the UL112–113 promoter driving expression of the CAT reporter was inserted between the US9 and US10 loci in the viral genome. Reporter expression from this ectopic location authentically reproduced the kinetics of UL112–113 expression, in particular the switch from the +1 to the −62 transcription start site late in the infection (Staprans and Spector, 1986). The family of viruses was constructed based on the observations discussed above and comprised recombinants containing the wild type UL112–113 promoter or one of a series of IE2 86 and/or ATF/CREB binding site mutations in the promoter. As in transient assays, deletion of the ATF/CREB site in the virus resulted in a severe reduction in reporter activity early in the infection, but by 72 h p.i. wild type and ATF/CREB deletion mutants differed in reporter activity by less than twofold. Deletion of the ATF/CREB site also resulted in a shift of the late transcription start site downstream by the number of residues deleted. Consistent with data from transient transfection assays, when the IE2 86 binding site between −113 and −85 was deleted, the level of transcription from the mutant promoter was reduced to half of wild-type promoter levels at early times. Later in the infection, this level was not sustained, and extracts from IE2 86 binding site mutant-infected cells exhibited 15-fold less CAT activity than extracts from wild-type promoter virus-infected cells. These results support a model in which transcription from the UL112–113 promoter is differentially controlled at early and late times postinfection, with the ATF/CREB site providing significant regulatory control at early times and little to none at late times. The IE2 86 binding site, in contrast, modulates UL112–113 transcription at early times but is even more important for the maintenance of elevated transcript levels late in the infection.

Further mutational analysis of the UL112–113 promoter was used to define the sequences between −113 and −85 involved in the control of late transcription. Insertion of 5- or 10-nt sequences into the promoter and a corresponding shift in the late transcription start site suggest that sequences in this region direct late transcription from the UL112–113 promoter with distance-dependent characteristics (D. Kim and D. H. Spector, unpublished results). This complex control of transcription from the UL112–113 region reinforces the idea that, while overall expression of a given viral gene may appear to change little as the infection progresses, this steady-state level is achieved through a series of temporally distinct regulatory mechanisms.

In addition to the above controls operating at the level of transcription, analysis of the pattern of expression of the four RNAs and their corresponding proteins revealed that there were additional mechanisms being used to regulate the level of the proteins (Wright and Spector, 1989). The high levels of the 43 kDa UL112–113 protein at early times correlated well with the abundance of the 2.2 kb RNA at this time. The kinetics of synthesis of the 84- and 34-kDa proteins also correlated well with those of their corresponding RNAs. In contrast, the level of the 2.1 kb RNA was only slightly lower than that of the 2.2 kb RNA at all times during the infection, but the 50 kDa protein did not accumulate until later in the infection. Interestingly, the level of the 50 kDa protein was most sensitive to inhibition of viral DNA replication, suggesting that its accumulation at late times might be coupled to ongoing viral DNA replication. The mechanism for this post-transcriptional regulation is still unknown. It does not appear to be related to stability of the full-length protein, as in pulse-chase experiments at early times in the infection, the 50 kDa protein showed similar kinetics of decay as the 43 kDa protein. Other possibilities are that there is some block to efficient translation of the RNA or that the protein is transiently unstable during translation.

Multiple *cis*-acting sequences regulate UL54 expression

UL54, the ORF encoding the HCMV DNA polymerase, is a prototypical early–late gene whose regulation has been studied both in the context of the viral genome and in numerous transient expression and in vitro assays

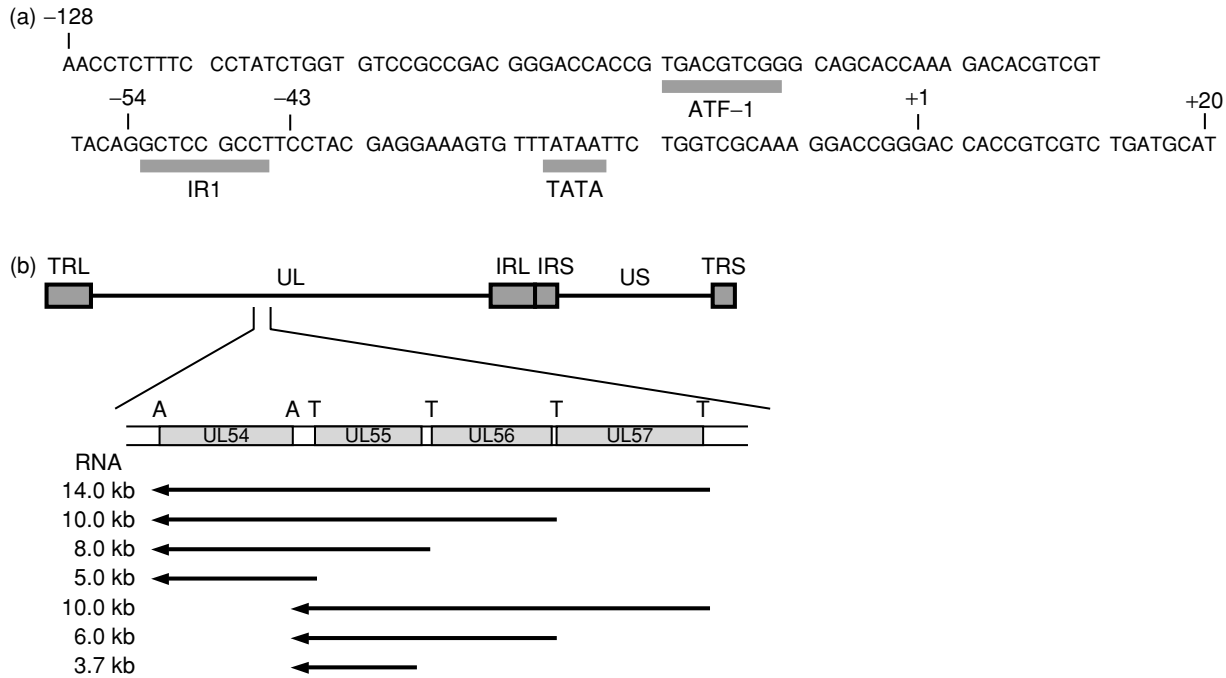

Fig. 18.3. Structure of the HCMV UL54 region. (a) The minimal UL54 promoter from nt −128 to +20 relative to the transcription start site, +1. The IR1 element located between −53 and −45 and the ATF-1 binding site from −88 to −80 are indicated. Sequences between nt −54 and −43 are required for IE protein-mediated promoter activation. (b) Transcripts expressed from the UL54-UL57 region. At least seven RNAs resulting from transcription from four start sites (predominant TATA elements, T) and polyadenylation at two sites (predominant poly A sites, A) are expressed from this region.

(Fig. 18.3). Seven transcripts resulting from initiation at four sites and polyadenylation at two sites in the UL54–57 region are expressed from this cluster of genes. The first RNA transcripts containing UL54 sequences are detectable as early as 8–12 h p.i., but in contrast to the UL112–113 family of transcripts, their level increases significantly at later times (Smuda *et al.*, 1997).

Initial transient expression studies on the regulation of the UL54 promoter indicated that IE2 86 was required for its activation and that other IE and early products including IE1 72, TRS/IRS1, and the UL112–113 proteins cooperated to further activate transcription from the promoter (Kerry *et al.*, 1996). These experiments also identified promoter elements required for activation in transient and in vitro assays, defining the minimal polymerase promoter as the region from −128 to +20 relative to the transcription start site and demonstrating that an 8 bp inverted repeat element at −53 to −45, IR1, was required for activation of the polymerase promoter by viral IE proteins in transient transfection assays (Kerry *et al.*, 1994, 1996). A second copy of the IR1 element is present at −225, but did not contribute significantly to activation of the polymerase promoter in these assays (Kerry *et al.*, 1996). IR1 was found to bind cellular factors found in nuclear extracts prepared from infected cells. If the IR1 element was mutated, the cellular factors failed to bind and activation of the promoter by IE proteins was decreased threefold (Kerry *et al.*, 1994). A pair of studies identified the transcription factor Sp1 as one of the cellular proteins that could bind the IR1 element. In one study (Luu and Flores, 1997), scanning mutagenesis of the promoter from −270 to +200 confirmed the requirement of the −54 to −43 region for IE mediated activation of the promoter. Using a 30 bp DNA probe and uninfected HeLa cell extracts, the authors demonstrated that Sp1 bound to this region. In the other study (Wu *et al.*, 1998), a shorter IR1-containing DNA probe also bound Sp1 in extracts of U373 cells overexpressing IE2 86, but not in parental U373MG cells or in HeLa cells. The complex could be supershifted by the addition of antibody specific to IE2 86, but the presence of IE2 86 in this complex did not seem to require DNA binding activity since addition of an unlabeled DNA probe containing the *crs* did not reduce complex formation. In addition, an Sp1 binding oligonucleotide competed away this complex in the IE2 86-expressing U373 cells. Based on these results, the authors proposed that an inhibitory factor present in HeLa cells prevents the Sp1-UL54 promoter interaction from occurring.

Numerous differences in experimental design prevent further direct comparison of these studies, and the role of Sp1 in control of UL54 expression in infected cells has not yet been examined.

Another regulatory domain in the UL54 promoter was localized to between nt −88 to −80 (Kerry *et al.*, 1994, 1997). A 40 bp DNA probe from this region bound nuclear proteins from infected human fibroblasts, with DNA binding activity particularly strong in 48–72 h p.i. extracts and weaker at earlier times or when DNA replication was inhibited by the addition of phosphonoacetate (PAA). Supershift analyses confirmed that one protein present in this complex is ATF-1. Since recombinant ATF-1/DNA complexes migrate differently than those present in infected cell nuclear extracts, it is possible that an additional protein is involved in this interaction or that ATF-1 is differentially modified during the infection.

Analysis of the UL54 promoter in the context of the viral genome began with the characterization of this promoter driving a CAT reporter in a construct inserted between ORFs US9 and US10 (Kohler *et al.*, 1994). Subsequent studies used similar recombinant viruses to better define the role of the IR1 element in UL54 promoter activation. A family of viruses was constructed in which either the full-length polymerase promoter (−425 to +20), the full length promoter with a mutation in the IR1 site, or the minimal activation domain (−128 to +20) drove expression of the CAT reporter (Kerry *et al.*, 1996). Based on CAT activity and RNA levels by Northern blot, UL54 promoter activity at early times was three- to fourfold lower than the wild-type when the IR1 element was mutated. In contrast, deleting the upstream promoter region and including only the minimal promoter resulted in a slight increase in promoter activity. This was consistent with the increase in promoter activity observed in transient assays when the −425 to −128 region was deleted, but the effect was smaller in the context of the viral genome. At late times, the IR1 element appears to be less important to the activation of this promoter, since the virus carrying the IR1 mutation exhibited only a slight reduction in both RNA levels and CAT activity by 72 to 96 h p.i. The ATF site in the UL54 promoter was similarly examined by constructing recombinant viruses (Kerry *et al.*, 1997). In contrast to the IR1 element, the ATF binding site appears to control UL54 promoter activity both at early and late times p.i. Mutation of the ATF site in a UL54 promoter-CAT reporter virus resulted in five- to sixfold decreases in both mRNA expression and CAT activity over a range of times, from 24–96 h p.i.

Finally, recent work has attempted to use regulation of the UL54 promoter to understand the species specificity of HCMV (Garcia-Ramirez *et al.*, 2001). These experiments examined the activity of the HCMV polymerase promoter following HCMV infection of murine NIH 3T3 cells transiently or stably transfected with a yeast artificial chromosome (YAC) clone containing much of the HCMV genome. The authors found that mutating the IR1 element in this context reduced reporter activity and interpreted these results to invoke similar cellular factors in both 3T3 and human cells, suggesting that species specific cellular factors do not underlie activation of the UL54 promoter. Given the many differences between murine and human fibroblasts, it is difficult to put these findings in context. A complete characterization of the UL54 promoter-reporter YAC construct in human cells to understand the consequences of promoter mutations in the natural context might help understand any work across species barriers.

UL4 expression is controlled at the transcriptional and translational levels

UL4 is an example of an early gene whose expression is regulated by unique mechanisms operating at both the transcriptional and translational levels (Fig. 18.4). Three unspliced transcripts are expressed from the UL4 locus, with 1.4 and 1.5 kb RNAs present at early times and a 1.7 kb product detectable later. These encode an incompletely characterized membrane glycoprotein, gp48, which is a virion component that is completely dispensable for viral growth in tissue culture (Hobom *et al.*, 2000).

Transcriptional control of the UL4 gene was first analyzed in a series of transient transfection assays demonstrating that the UL4 promoter is responsive to IE2 86 and that there are two *cis*-acting sites in the UL4 promoter upstream of the TATA box (Chang *et al.*, 1989; Huang *et al.*, 1994). The first site, a CCAAT box with inverted dyad symmetry between −88 and −98 relative to the transcription start site, appeared to be a positive regulatory element that could bind the transcription factor NF-Y in vitro (Huang *et al.*, 1994). Gel shift analyses demonstrated the formation of two complexes that were inhibited by the addition of a competing oligonucleotide containing the NF-Y target sequence or supershifted by the addition of antibody to NF-Y. Neither could be supershifted by the addition of IE2 86-specific antibody. One complex was present in both uninfected and infected cells; the second was only detected in infected cells. When phosphatase was added, the second complex decreased in abundance while the first increased, suggesting that NF-Y may be differentially phosphorylated in HCMV-infected cells.

A second regulatory region located between −169 and −139 in the UL4 promoter, site 2, bound a cellular factor in

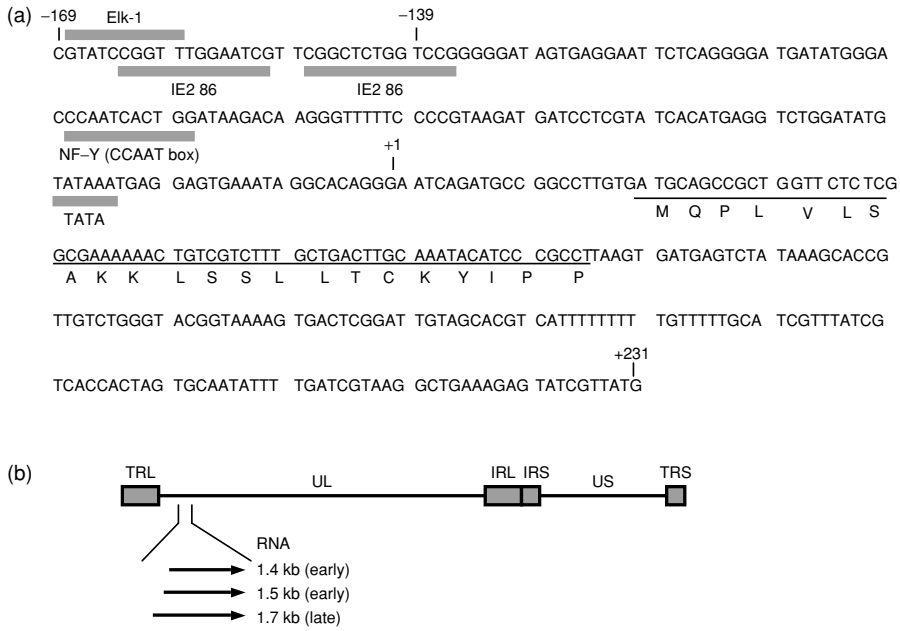

Fig. 18.4. Structure of the HCMV UL4 region. (a) The UL4 promoter and transcript leader sequences from nt −169 to the start site of gp48 (pUL4) translation. Sequences are representative of HCMV Towne strain and are numbered relative to the start site of 1.5 kb RNA transcription, +1. Site 2 is located between −169 and −139 and contains two putative IE2 86 binding sites and the Elk-1 site. The CCAAT box binds NF-Y. uORF2, which encodes the *cis*-acting repressor of UL4 transcription, is underlined, and the sequence of the 22 aa peptide is indicated. (b) Transcripts expressed from the UL4 region. Three 3′ coterminal transcripts with different 5′ ends are produced. Two RNAs of 1.4 and 1.5 kb are present at early times, with the 1.5 kb transcript expressed more abundantly. The 1.7 kb transcript is present later in the infection.

vitro, as indicated by DNA footprinting and gel shift assays, and negatively influenced transcription (Huang *et al.*, 1994; Huang and Stinski, 1995). The importance of IE2 86 in the activation of this promoter was suggested by the following observations: first, the negative effect on the promoter was relieved when an IE2 86 expression vector was cotransfected with the UL4 promoter–CAT reporter construct; second, a truncated version of IE2 86 containing amino acids 290 to 579 bound site 2 in vitro (Huang and Stinski, 1995). These results led to the hypothesis that IE2 86 binds to a specific negative regulatory region (which is 65% homologous to the *crs* element in the major IE promoter) and in the process displaces a bound cellular factor, thus allowing activation of the UL4 promoter. Mutation of the putative zinc finger region of IE2 86 resulted in a protein that could no longer interact with the promoter, lending further support to this idea.

Recent studies examining transcriptional control of the UL4 promoter in the context of the viral genome confirm some, but not all, of these initial findings and demonstrate important differences between control of viral gene expression in transient assays and in the infected cell. A series of HCMV recombinants containing the CAT reporter cassette driven by a wild type or mutant UL4 promoter inserted into the viral genome between US7 and US12 were derived (Chen and Stinski, 2000). In this ectopic location, the CAT transcript was expressed with kinetics much like those of the native UL4 transcript, but to slightly lower overall levels. Here, a mutation in the UL4 promoter NF-Y binding site did not alter CAT transcript levels, leading to the conclusion that in the virus, the NF-Y binding site contributes little or nothing to control of UL4 transcription. In contrast, site 2 mutants displayed altered reporter expression. Site 2 contains two putative (but non-consensus) IE2 86 binding sites and a predicted Elk-1 binding site that together appeared to be required for maximal expression in the context of viral infection (Chen and Stinski, 2000). In a later study, however, disruption of the Elk-1 site alone was sufficient for the effect, indicating that it is likely Elk-1 or a related cellular factor and not the IE2 86 binding sites that play the critical regulatory role (Chen and Stinski, 2002). Elk-1 binding to this site is supported by EMSA data in which a consensus Elk-1 site competes for cellular factor binding and an Elk-1 antibody partially supershifts the UL4 promoter-cellular factor complex (Chen and Stinski, 2000).

Transcriptional upregulation of UL4 expression has been used to demonstrate the importance of the MAPK/ERK and p38 MAPK pathways in HCMV replication. Peak activation of these signal transduction cascades occur at 4 and 8 h p.i., respectively, and appears to be important to the progression of productive infection via efficient expression of early and late genes (Johnson *et al.*, 2001a; Rodems and Spector, 1998). In one study (Chen and Stinski, 2002), the requirement for these pathways was analyzed in the context of UL4 transcription using the MAPK/ERK kinase (MEK) inhibitor UO126 and the p38 MAPK inhibitor FHPI. Cells infected with one of the recombinant viruses expressing the CAT gene from wild-type or mutant UL4 promoters were treated with an inhibitor and assayed for CAT activity. Strikingly, inhibition of MEK with the chemical inhibitor UO126 caused UL4 promoter activity to drop by 70%–80% for all promoters tested, with approximately equivalent loss of expression when the wild-type UL4 promoter was used as well as when site 2 or the NF-Y or Elk-1 binding sites were disrupted. The effect of the inhibitor FHPI was very similar, with no effect on UL4 promoter constructs when used at a low concentration and a 50%–80% reduction of both wild type and mutant promoter activity at a higher concentration. These data suggest that the UL4 promoter is responsive to both MAPK/ERK and p38 MAPK pathways and that the minimal responsive element is the TATA, not one of the other transcription factor binding sites that have been analyzed. This effect, however, was less prominent when endogenous UL4 RNA expression was examined, and the possibility of general inhibition of viral transcription or downregulation of other upstream factors in the presence of these inhibitors has not yet been addressed.

Novel post-transcriptional controls also distinguish the regulation of UL4 expression. Three small ORFs are located in the UL4 transcript upstream of the gp48 transcription start site. The largest of these, uORF2, encodes a 22 aa product which acts in *cis* to repress translation of the authentic UL4 transcript (Degnin *et al.*, 1993). Interestingly, this repression is amino acid- (but not nucleotide-) sequence dependent and is particularly sensitive to mutations in codons near the C-terminus of uORF2. In transient transfection-superinfection assays, missense mutation or deletion of the terminal amino acid, a proline, resulted in significantly elevated expression of reporter protein while the level of UL4 promoter-driven transcript remained unchanged. Changes in the N-terminal leader sequence also had an effect, with missense mutations at codons 7 and 8 reducing the ability of uORF2 to block translation. A further series of modified primer extension assays showed that ribosomes are stalled on the RNA at the uORF2 termination codon. These data have led to the following hypothesis regarding the mechanism of inhibition of downstream translation by uORF2. A ribosome translates through the uORF2 sequence until it reaches the final proline codon. Here, the nascent uORF2 peptide remains covalently linked to the peptidyl-tRNA and bound to the ribosome on the transcript. A possible interaction between this complex and the release factor eRF1, mediated by C-terminal prolines at positions 21 and 22 in the uORF2 peptide and a GGQ motif in eRF1, stabilizes the intermediate and prevents hydrolysis of the peptidyl-tRNA bond (Cao and Geballe, 1996, 1998; Janzen *et al.*, 2002). Ribosomes stall before they are able to reach the gp48 translation start site, resulting in uORF2-dependent repression of gp48 translation.

The above model is supported by results from recombinant viruses as well. When the uORF2 initiation codon in the UL4 region was mutated, there was a significant upregulation of gp48 protein expression by the mutant relative to the wild-type virus (Alderete *et al.*, 2001). In vivo, ribosomes were also stalled at the uORF2 termination codon in wild-type but not mutant-infected cells. The relatively normal expression of other early proteins indicates that it is not a general upregulation of translation in mutant-infected cells that accounts for this phenotype. Curiously, it appears that there is a slight delay in the accumulation of the UL4 transcript in mutant infected cells, with the UL4 RNA detectable by Northern blot 24 h p.i. in cells infected with the parental wild-type virus, but not until 48 h p.i. in uORF2 mutant-infected cells.

Since neither loss of nor overexpression of UL4 appears detrimental to viral replication in cultured fibroblasts (Alderete *et al.*, 2001; Hobom *et al.*, 2000), it seems likely that this complex regulation is important in the infected host where gp48 may have a cell type-specific role. Control of its transcription and translation remain interesting illustrations of the diverse methods used by the virus to regulate gene expression.

Human herpesviruses 6 and 7

Human herpesvirus 6 is distinguished by its ability to grow in T-lymphocytes. There are two variants: HHV-6A (GS and U1102-like isolates) and HHV-6B (Z-29 and HST-like isolates). Although the variants have approximately 90% nucleotide sequence identity and share many properties, they differ with respect to their epidemiology, cell tropism, interaction with the host cell, and in vivo pathogenesis. HHV-6B infection is very common in early childhood and is associated with exanthem subitum (roseola infantum), a mild illness that lasts only a few days (Yamanishi

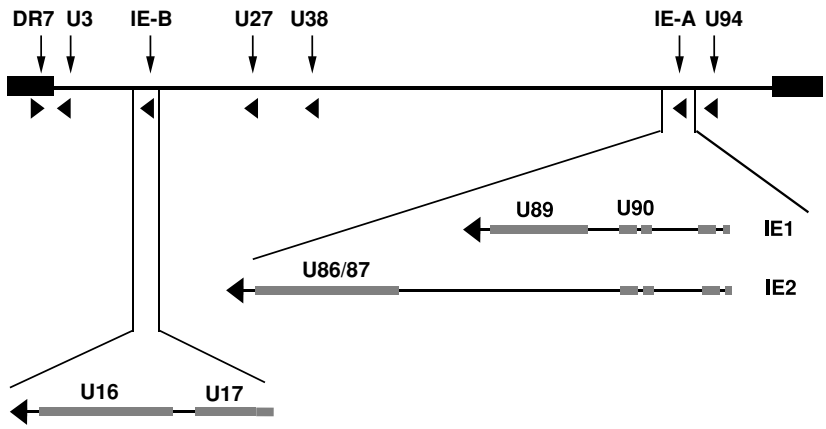

Fig. 18.5. HHV-6A Genome. The HHV-6 gene products with regulatory activities – DR7, U3, IE-B, U27 (DNA polymerase processivity factor), IE-A, and U94 – are shown on the map. The location of the U38 (DNA polymerase) ORF is also shown. The solid arrowheads indicate the direction of transcription. An expanded view of the IE transcripts encoded by the IE-A and IE-B loci is shown below the genome. The dark boxes are coding exons, the lighter boxes are non-coding exons, and the thin lines between the boxes are introns.

et al., 1988). HHV-7 is biologically similar to the two HHV-6 variants and is less frequently associated with this illness (Dominguez *et al.*, 1999; Isegawa *et al.*, 1999; Megaw *et al.*, 1998; Nicholas, 1996; Tanaka *et al.*, 1994). The DNA genomes of HHV-6 and HHV-7 are smaller than that of HCMV, with the length 159 kbp for HHV-6A, 161–170 kbp for HHV-6B, and 145–153 kbp for HHV-7 DNA. The genome of all of these viruses consists of a long unique segment bounded by direct repeats (Dominguez *et al.*, 1999; Gompels *et al.*, 1995; Isegawa *et al.*, 1999; Lindquester and Pellett, 1991; Martin *et al.*, 1991b; Megaw *et al.*, 1998; Nicholas, 1996)). In general, the genes of these viruses are collinear with those of human cytomegalovirus U_L region (see Chapter 14).

Analysis of the molecular biology of HHV-6 and HHV-7 has been limited by poor growth and low yields in cultured cells. Thus, the elucidation of the regulation of gene expression is not well advanced and has relied almost exclusively on transient assays that are known to be relatively poor predictors of behavior in the context of viral infection. The overall pattern of gene expression (IE, early, and late) resembles that of HCMV and other herpesviruses, but the assignment of many of the individual genes to a particular class is still tentative and has been influenced by the sensitivity of the assay applied, the specific strain of virus, and cell line used in the analysis. The reader is referred to Chapters 42 and 43 as well as the following papers for the details: Menegazzi *et al.* (1999); Mirandola *et al.* (1998); Oster and Hollsberg (2002); Rapp *et al.* (2000). For the purposes of this review, only HHV-6 IE genes that appear to have some regulatory functions and two early genes whose regulation has been studied in greatest depth – the HHV-6 DNA polymerase (U38) and the DNA polymerase processivity factor (U27) (Fig. 18.5) – will be discussed.

HHV-6 IE gene products with regulatory activities

The HHV-6 regulatory proteins that have been studied are encoded by the IE-A and IE-B loci, DR7, U3, U27, and U94. The IE-A and IE-B loci of both HHV-6 and HHV-7 are the major sites of IE gene expression (Fig. 18.5). The IE-A region (U86 to U90) is collinear with the major immediate early region of HCMV that encodes the IE1 and IE2 proteins (UL122–123), and the IE-B region (U16 to U19) is collinear with the HCMV IE ORFs UL36 to UL38 (see Chapter 17).

These putative viral regulatory proteins have only been studied with promoters from cellular genes and genes from heterologous viruses. Because HHV-6 has been proposed to be a cofactor in the progression of AIDS, many of these studies have used the HIV-1 LTR promoter (for review, see Lusso and Gallo, 1995). The HIV-1 LTR has been found to be activated by regions of the HHV-6A genome corresponding to the IE-B locus (Chen *et al.*, 1994; Garzino-Demo *et al.*, 1996; Geng *et al.*, 1992; Horvat *et al.*, 1991), the IE-A locus (Gravel *et al.*, 2003; Martin *et al.*, 1991a; Papanikolaou *et al.*, 2002; Stanton *et al.*, 2002), DR7 (Kashanchi *et al.*, 1994; Thompson *et al.*, 1994a), U3 (Mori *et al.*, 1998), and U27 (Zhou *et al.*, 1994). The IE-A locus also transactivates the CD4 promoter (Flamand *et al.*, 1998) and adenovirus E3 and E4 promoters (Martin *et al.*, 1991a), and the IE-B locus activates HPV 16 and 18 promoters (Chen *et al.*, 1994). Some of the regulatory proteins also appear to have negative effects. For example, U94 negatively regulates HIV-1 and H-ras promoters (Araujo *et al.*, 1995), and DR7 negatively regulates

promoters that are responsive to p53 (Kashanchi *et al.*, 1997).

IE-A

HHV-6A and HHV-6B exhibit a different organization in the IE-A region (Dominguez *et al.*, 1999; Isegawa *et al.*, 1999). The HHV-6A IE-A locus consists of two genetic units that are referred to as IE1 and IE2 (Chapter 17). Although this region is collinear with the HCMV major IE locus UL123 (IE1 72) and UL122 (IE2 86), it shares no nucleotide or protein sequence homology. The IE1 region encodes a 3.5 kb transcript that consists of 4 small 5′ exons and a large 3′ exon (Fig. 18.5) (Schiewe *et al.*, 1994). Translation begins in exon 3, yielding a major protein of approximately 150 to 170 kDa that contains ORFs U90 and U89 (Papanikolaou *et al.*, 2002). The corresponding region of HHV-6B shares only 62% identity at the amino acid level and encodes a major IE protein of approximately 150 kDa (Gravel *et al.*, 2002; Takeda *et al.*, 1996). The proteins are phosphorylated and conjugated to the ubiquitin-like protein SUMO-1 (Chang and Balachandran, 1991; Gravel *et al.*, 2002). The sequence divergence of the HHV-6A and HHV-6B IE1 protein is reflected in their function in that the HHV-6A IE1 protein is a stronger activator of heterologous promoters in transient expression assays than the corresponding HHV-6B protein (Flamand *et al.*, 1998; Gravel *et al.*, 2002; Martin *et al.*, 1991a).

Both HHV-6A and HHV-6B IE1 proteins are able to traffic to the ND10 sites (PODs). However, in contrast to other herpesviruses, interaction of the ND10 domains with IE1 does not lead to their dispersal, and IE1 maintains a stable interaction with ND10 throughout the infection (Gravel *et al.*, 2002; Stanton *et al.*, 2002). By immunostaining, the highest number of individual IE1 bodies can be detected at 12 h p.i., when they begin to coalesce into 1–3 larger bodies. It is likely that other viral proteins are needed to generate the larger bodies, since when the IE1 proteins were individually expressed, they colocalized with PML and SUMO-1 but the ND10 sites did not coalesce. Thus as is the case for HCMV, dispersal of ND10 domains is not a prerequisite for productive infection.

Recently, a full-length cDNA encoding the HHV-6A IE2 protein was isolated (Gravel *et al.*, 2003); it contains ORFs U90 and U86/87, the positional homologues of the HCMV ORF encoding IE2 86 (Fig. 18.5). The 5.5 kb IE2 mRNA is expressed in the absence of *de novo* protein synthesis with kinetics that are somewhat delayed relative to the IE1 transcript. The IE2 mRNA is also less abundant, but it continues to increase throughout the infection while the IE1 mRNA reaches maximal levels by 12 h p.i. At later times, larger transcripts that have not been characterized and may initiate from upstream sites appear. The processed IE2 mRNA consists of 5 exons; 4 small exons located upstream of U89 are shared with IE1 and the fifth exon corresponds to the IE2 specific ORF U86/87. The sequence of the HHV-6A IE2 transcript diverges from that encoded by the HHV-6B variant, with only 64% identity at the amino acid level. The HHV-6A IE2 protein is also 167 amino acids shorter. By Western blot analysis with an antibody specific for the IE2 region, it appears that the major IE2 protein is approximately 220 kDa. At later times in the infection, additional smaller proteins of 100, 85 and 55 kDa have also been observed (Gravel *et al.*, 2003; Papanikolaou *et al.*, 2002). Analogous to the HCMV IE2 86 protein, the full-length HHV-6A IE2 protein functions in transient expression assays as a promiscuous activator of multiple promoters, including minimal promoters containing only a TATA box (Gravel *et al.*, 2003; Papanikolaou *et al.*, 2002). Recently, deletion analysis showed that both the N- and C-terminal domains of this protein are required for full function (Tomoiu *et al.*, 2006). Interestingly, the HHV-6A IE2 protein does not appear to downregulate its own promoter, possibly because it does not contain the HCMV *cis*-repression signal.

IE-B

The HHV-6 IE-B region consists of the ORFs U16, U17, U18, and U19 and is collinear with the HCMV region containing the UL36–38 ORFs. As shown for the HCMV UL36–38 region, transcription from the HHV-6 IE-B region is complex (Flebbe-Rehwaldt *et al.*, 2000; Mirandola *et al.*, 1998). In HHV-6A (GS strain), the U17 and U16 ORFs are positional homologues of HCMV UL36 and yield a spliced IE RNA (Fig. 18.5) (Flebbe-Rehwaldt *et al.*, 2000; Mirandola *et al.*, 1998). HCMV UL36 also consists of 2 exons, with exon 2 corresponding to HHV-6A U16 (Tenney and Colberg-Poley, 1991a, b). In addition to the HHV-6 transcripts that contain the U17/U16 ORFs, there are also transcripts that include the U16 and U15 ORFs (U15 is unique to HHV-6). The HHV-6A and HHV-6B U17/U16 RNAs first appear at IE times and are maintained throughout the infection. The other U16 containing transcripts are expressed primarily as early RNAs, although a low level of transcription can be detected at IE times.

The HHV-6 U18 and U19 ORFs are transcribed as multiply spliced early RNAs. These are positional homologues of the HCMV UL37 and UL38 genes, respectively, but may not be functional homologues. With the caveat that all of the functional assays have been performed with transient assays and plasmid constructs, the ORFs do not correspond to their HCMV homologues with respect to their potential role as transactivators. In HHV-6, the ORFs that can

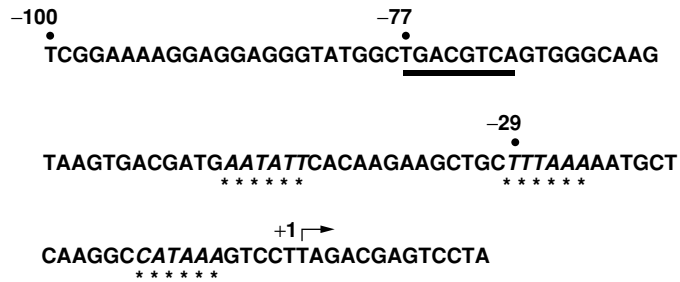

Fig. 18.6. HHV-6A DNA polymerase (U38) promoter. Sequences that could potentially serve as TATA boxes are italicized and marked with asterisks. The arrow at position +1 shows the transcription start site. The consensus ATF/CREB binding site is underlined.

transactivate the HIV-1 LTR promoter are U17/U16, not U18/U19 (Chen *et al.*, 1994; Garzino-Demo *et al.*, 1996; Geng *et al.*, 1992; Horvat *et al.*, 1991). UL37 exon 1, which has no apparent homologue in HHV-6 or HHV-7, serves as a cell death suppressor and transactivator of HCMV early gene promoters in transient assays (Colberg-Poley *et al.*, 1998; Patterson and Shenk, 1999; Goldmacher *et al.*, 1999).

U94

U94 is encoded by HHV-6A and HHV-6B and is a homologue of the adenovirus associated virus 2 (AAV-2) rep gene (Thomson *et al.*, 1991). Although not conserved in primate CMVs, a homologue is found in rat CMV (Vink *et al.*, 2000). In AAV-2, the rep68 gene encodes a site-specific ATP-dependent endonuclease and helicase that is involved in the site-specific integration of AAV into chromosome 19. U94 can serve as a helper for AAV-2 replication and can complement an AAV-2 virus with a mutation in the rep gene (Thomson *et al.*, 1994). The role of the U94 protein is yet to be elucidated, but it may play a role in latency and modulation of the infection. Relevant to this are the data showing that cell lines expressing HHV-6B U94 cannot be infected by HHV-6A and that the U94 transcript can be detected during latency in PBMC (Rotola *et al.*, 1998). U94 was also found to suppress H-ras and BPV-1 transformation (Araujo *et al.*, 1997) and transcription from H-ras and HIV-1 LTR promoters (Araujo *et al.*, 1995). HHV-7 does not encode a U94 homologue.

cis-acting sequences within HHV-6 early promoters

DNA polymerase (U38)

Analogous to HCMV, the HHV-6 DNA polymerase gene has been used as a prototypical early gene for examining *cis*-acting regulatory sequences on the promoter (Fig. 18.6). Based on the position of the RNA start site, standard transient expression assays with promoter-CAT reporter genes have been used to identify and characterize the major regulatory region for transcription (Agulnick *et al.*, 1994). The data showed that expression of CAT from a construct containing sequences −524 to +115 relative to the transcription start site was below the limit of detection in uninfected HSB-2 human T cells, but was highly up-regulated in infected cells. By mutational analysis, the cis-acting sequences for activation were localized to the sequences between −78 and +13. The major regulatory element within this region was a consensus ATF/CREB binding site located at nt −77 to −70. Further support for this domain serving a regulatory role was the observation that in gel shift assays, this site bound to two protein complexes in both infected and uninfected nuclear extracts. The ATF/CREB site, however, is not required for activation of this promoter by the HHV-6A IE2 protein in transient assays (Tomoiu *et al.*, 2006).

The U38 promoter does not contain a consensus TATA element in the –30 region, although there are several AT-rich domains located at positions −48 to −43, −29 to −24, and −11 to −6. When point mutations were introduced into each of the domains individually, there was no effect on the promoter, leading the authors to conclude that the promoter is TATA-less (Agulnick *et al.*, 1994). However, the start site for transcription was not identified for any of the mutants in the transient expression assays, and thus the possibility that the elements might be able to compensate for one another, albeit with a change in start site, cannot be excluded. As has been the case for all herpesvirus promoters, conclusions cannot be drawn from transient assays alone and each promoter must be assessed in the context of the viral genome. It would seem likely that the ATF/CREB site is the major regulatory element, as this sequence is located in a similar position in the promoter of the HCMV DNA polymerase gene and its function has been confirmed in the context of the HCMV genome (Kerry *et al.*, 1994, 1996, 1997).

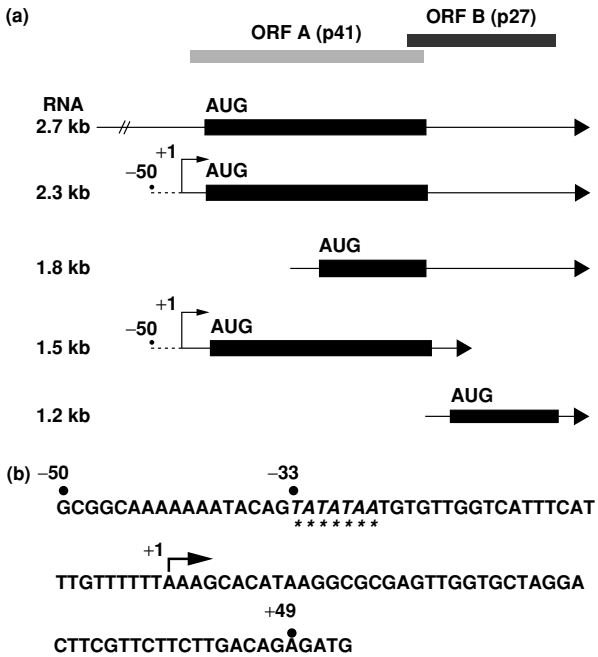

Fig. 18.7. HHV-6A DNA polymerase processivity factor (U27). (a) The early–late transcripts from the U27 region are shown. ORF A encodes the 41-kDa DNA polymerase processivity factor. The dark boxes represent the putative protein product. (b) Promoter for the 1.5 kb and 2.3 kb U27 RNAs. The sequence that could potentially serve as a TATA box is italicized and marked with asterisks. The arrow at position +1 shows the transcription start site. The translation initiation codon at nt +49 is also indicated.

DNA polymerase processivity factor (U27)

U27 encodes a 41 kDa nuclear phosphoprotein that is the homologue of the HCMV UL44 gene encoding DNA polymerase processivity factor (Fig. 18.7) (Agulnick *et al.*, 1993; Chang and Balachandran, 1991). The U27 region actually includes two ORFs, ORF A and ORF B. ORF A corresponds to a 41 kDa protein, and the downstream ORF B could encode a 27 kDa protein (Zhou *et al.*, 1997). The transcription pattern of this gene is similar for both the variant A HHV-6 GS strain and the variant B HHV-6 Z29 strain. At least five early–late unspliced RNA species ranging in size from 1.2 to 2.7 kb (1.2, 1.5, 1.8, 2.3, and 2.7 kb) map to this locus, with the 2.3 kb RNA being the most abundant (Agulnick *et al.*, 1993; Zhou *et al.*, 1997). Four of the RNAs (2.7, 2.3, 1.8, and 1.2 kb RNAs) are 3′ co-terminal. The 1.5 kb RNA utilizes the same start site as the 2.3 kb RNA but terminates upstream of the other four RNAs. The 2.3 kb and 1.5 kb RNAs have the potential to encode the p41 protein in ORF A, and the 1.2 kb RNA could specify a protein of about 17 kDa within ORF B. Although the 2.7 kb RNA includes ORF A, the presence of several AUGs before ORF A makes it unlikely that it encodes p41. Likewise, the 1.8 kb RNA has several AUGs with short ORFs before ORF B, but it could be translated into a truncated version of ORF A.

Regulation of the promoter for the 1.5 kb and 2.3 kb U27 RNAs was studied for the HHV-6A GS strain in HSB-2 human T-cell line (Thompson *et al.*, 1994b). The transcription start site, which is 48 bp upstream of the translation initiation codon AUG, is preceded by a TATA sequence starting at nt −33 relative to the RNA start site. Using mutant promoter-CAT constructs in transient expression assays, an essential regulatory element that was activated in the infected cells was localized between nt −73 and −52. Within this region were a putative binding site for the transcription factor C/EBP (CAAT enhancer-binding protein) and two other repeat sequences. The activity of this site was both distance and orientation dependent relative to the TATA sequence.

Mobility shift assays indicated that there were four complexes that bound to this region. The two that were present in both uninfected and infected cells (C1 and C2) did not appear to contain C/EBP factors. Point mutations within these sites that eliminated binding also inactivated the promoter. Two of the binding complexes were only present in uninfected cells (C3 and C4) and they could be competed by an oligonucleotide containing a consensus C/EBP site. The construction and use of a HHV-6 BAC will greatly facilitate further studies on the regulation of this and other promoters during the viral life cycle.

Conclusions

The studies presented here highlight the central role that early gene expression plays in the viral life cycle. It is the middleman in the relay race leading to the production of infectious virus. Events precipitated by the initial contact of the virus with the host cell and the synthesis of IE gene products set the stage for early gene expression. The products of these early genes not only provide the component parts for the factories devoted to viral DNA synthesis, cleavage and packaging of the viral genome, and assembly of the virus particles, but also serve to commandeer the host cell machinery and signaling pathways to create a cellular environment that is optimal for viral gene expression and DNA replication. Although dispensable for growth in tissue culture, a cadre of the early genes must also establish a blockade to the host's immune response and prepare routes of escape for viral dissemination. Analogous to the control of host cell genes, viral early gene expression is regulated at multiple levels by mechanisms operating at the initiation of transcription, RNA processing and transport, translation,

and mRNA and protein stability. The development of BACs as vectors for the cloning of herpesvirus genomes has revolutionized the field so that the function of viral genes and the regulation of their expression can be studied in the biologically relevant context of the viral infection. These techniques coupled with the rapidly moving fields of genomics and proteomics will greatly enhance our ability to elucidate the cellular and molecular mechanisms governing the interaction of the virus with its host.

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DNA synthesis and late viral gene expression

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Overview

Much of the current understanding of betaherpesvirus DNA synthesis is based on studies with the cytomegaloviruses and is further shaped by comparison with prototypic alpha- and gammaherpesviruses. As for all herpesviruses, betaherpesvirus DNA synthesis occurs in the nucleus and relies on a core set of virus-coded proteins composing the replication fork machinery (detailed later) working together with *trans*-acting functions that promote initiation on a genetically defined, *cis*-acting replicator, called *ori*Lyt. DNA synthesis initiates in the vicinity of *ori*Lyt as soon as essential virus coded proteins appear, producing high molecular weight replication intermediates whose structure has not been fully characterized. Onset of viral DNA synthesis licenses transcription of a subset of the late class of viral genes, many of which encode proteins that assemble and constitute the complex virion. Subsequently, replication intermediates are resolved, and the progeny genomes are packaged into preformed capsids and mature ends are formed by the encapsidation machinery. Because these replication functions are essential for viral replication and pathogenesis, and differ from host counterparts, they have been candidate targets for the development of antiviral drugs. Moreover, further study of DNA replication and encapsidation may provide new insights about cellular components that contribute to these processes. Our goals in this chapter are to provide an up-to-date summary of betaherpesvirus lytic-phase replication machinery, to highlight emerging contrasts to other herpesviruses, and to consider how DNA synthesis-dependent late gene expression is regulated.

Soon after nuclear entry, a fraction of the input linear cytomegalovirus genomes circularizes by a process that does not require *de novo* protein synthesis, and these circular forms have been considered the likely template for subsequent transcription and replication events. In this model concatemeric products are generated from a rolling circle intermediate in much the same way this process is envisioned for other herpesviruses (Chapter 4). Unfortunately, the lack of any cell-free replication systems has left the process of DNA replication in any of the herpesviruses poorly understood. Consistent with a rolling circle model is the finding that *ori*Lyt-containing circular plasmids are replicated in virus-infected cells to form head-to-tail concatemers. Some models posit an initial theta form mode, but evidence is scant and there is no published evidence to support this possibility for any betaherpesvirus. Authentic HCMV replication intermediates contain very few circles and have structural characteristics consistent with the rolling circle model, but the majority are not readily resolved in monomeric form by digestion with unique-cutting restriction enzymes, arguing that more complex – probably branched – structures predominate, rather than simple concatemers or circles (McVoy and Adler, 1994). The observed abundance of apparently complex products might arise from redundant initiation, from the presence of recombination intermediates, or from strand invasion; however, unambiguous evidence for any of these mechanisms is lacking. Interestingly, HHV-6 produces concatemers with relatively little branching (Severini *et al.*, 2003), suggesting that whatever event(s) contributes to the complex replication intermediates seen in cytomegaloviruses and many other herpesviruses are not a requisite component of roseolovirus replication.

Recent, well-received evidence employing HSV-1 regulatory gene mutants supports a model for viral DNA synthesis beginning on linear genomes, and proceeding via branched intermediates rather than via circularization and rolling circle mechanisms. This work implies that circularization may actually suppress replication and promote establishment of latency (Jackson and DeLuca, 2003).

Available data do not exclude such a model for HCMV, because the inhibition of DNA synthesis by drug treatment from the time of infection results in the persistence of both circular and nuclease-sensitive linear HCMV genomes (McVoy and Adler, 1994). Given that circular genomes are associated with HCMV latency (Bolovan-Fritts *et al.*, 1999), these certainly appear to serve as templates for initiation of DNA synthesis upon reactivation. The extent to which the high molecular weight cytomegalovirus replication intermediates observed following infection of permissive cells arise preferentially by a rolling circle mechanism from circular templates or by some other mechanism will require further study.

Despite biological similarities between cytomegaloviruses and roseolaviruses, initiation of DNA replication proceeds with distinct mechanisms. The genomic location of the *cis*-acting element, *ori*Lyt is similar, being positioned between genes encoding the homologues of HCMV UL57 and UL69; however the sequence elements are very different. HCMV has a highly complex *ori*Lyt region that spans over a kilobase of DNA, whereas roseolaviruses rely on a simpler *ori*Lyt with structural similarities to alphaherpesviruses DNA replication origins (Chapter 10). Likewise, the *trans*-acting functions are distinct, with HCMV relying on transcriptional transactivators and roseolaviruses relying on an origin binding protein homologous to those encoded by alphaherpesviruses.

Betaherpesvirus replication proteins

A cotransfection–replication assay has enabled the identification of the subset of betaherpesvirus proteins required for origin-dependent DNA replication. In this assay, cloned viral genes are transfected along with a plasmid containing *ori*Lyt. When all *trans*-acting functions are included, amplification of the *ori*Lyt-containing plasmid occurs. Amplification of *ori*Lyt is detected using the restriction enzyme *Dpn* I, which removes bacterially propagated plasmid DNA due to cleavage specificity for methylated adenine. Input, nonreplicated DNA, is thereby removed and subsequent DNA blot hybridization detects specifically replicated DNA. This assay identified HSV-1 replication proteins (Wu *et al.*, 1988) and eleven essential loci contributing to the amplification of HCMV *ori*Lyt (Pari and Anders, 1993). The genes and their protein designations are listed in Table 19.1. HCMV DNA replication requires the same set of core replication proteins as HSV-1. Later studies, performing cotransfections using plasmids encoding the replication proteins under the control of strong constitutively active promoters, showed that one subset of these genes was directly involved in the enzymatic activity of DNA synthesis whereas other genes had roles in gene regulation or cell death suppression (Sarisky and Hayward, 1996). It was determined that one protein, UL84, appeared to be unique among the HCMV replication proteins together with a requirement IE2 in human fibroblast cells (Pari and Anders, 1993; Sarisky and Hayward, 1996).

Table 19.1. HCMV genes required for *ori*Lyt-dependent DNA replication

HCMV genes	HHV6 genes	Proposed function
UL44	U27	Polymerase accessory protein
UL54	U38	Polymerase
UL57	U41	Single-stranded DNA binding protein
UL70	U43	Primase
UL84		Early protein with UTPase and nucleic and binding activity
UL102	U74	Primase-associated factor
UL105	U77	Helicase
IE2		Transactivator; along with UL84 activates *ori*Lyt promoter
UL36–38		Auxiliary functions
UL112–113		Auxiliary functions
IRS1/TRS1		Auxiliary functions

HCMV DNA replication has characteristics that distinguish it from HSV-1, as well as from the roseolaviruses. Divergence includes a distinct type of origin binding protein (OBP) for each type of virus. HSV-1 encodes an OBP (Olivo *et al.*, 1988; Wu *et al.*, 1988) that initiates DNA synthesis by interacting with specific DNA sequences within *ori*Lyt (Chapter 10) and has associated enzymatic activities consistent with a role in initiation such as helicase activity (Bruckner *et al.*, 1991; Hazuda *et al.*, 1991). Both HHV-6B and HHV-7 encode an OBP that may function similar to that encoded by the alphaherpesvirus HSV-1. Roseolaviruses have *ori*Lyt sequences with OBP sites and other apparent similarities (Dewhurst *et al.*, 1994; Krug *et al.*, 2001) to HSV-1, including a role in transient replication (Dewhurst *et al.*, 1994; Dykes *et al.*, 1997). These origins are not similar in any way to HCMV *ori*Lyt or replication origins of other cytomegaloviruses. HCMV relies on IE2 and UL84 as apparent OBPs and both are betaherpesvirus-conserved proteins (Chapter 15); however, testing a role in cytomegalovirus replication awaits development of a suitable assay. What follows is a description of each HCMV replication protein and the current status with respect to proposed function.

Helicase–primase complex (UL105, UL70 and UL102)

As in all herpesviruses the HCMV helicase–primase is a heterotrimeric complex composed of a helicase subunit

(UL105), a primase subunit (UL70) and a linking subunit (UL102). In vitro assays determined that the three proteins interact as a complex (McMahon and Anders, 2002). The proposed helicase for HCMV was first identified as the homologue for HSV-1 UL5. The UL105 gene encodes a 3.4 kb transcript that is present in infected cells as early as 24 hours post-infection and a 110 kDa protein was detected using UL105 specific antiserum (Smith *et al.*, 1996). With respect to UL102, initial controversy regarding sequence data suggested that UL102 was a spliced transcript. Subsequent studies involving the identification of the UL102 mRNA isolated from an HCMV cDNA library indicated that UL102 is an unspliced 2.7 kb transcript which encodes an 873 codon ORF (Smith and Pari, 1995). The UL70 ORF encodes a 945 amino acid protein that is the proposed homologue for HSV-1 UL52. Like the HSV-1 primase, pUL70 contains a putative DXD motif that is common to the metal binding site found in prokaryotic primases and family B DNA polymerases (Dracheva *et al.*, 1995; Ilyina *et al.*, 1992; Li *et al.*, 1993). To date, no published study has demonstrated helicase or primase activity using recombinant HCMV proteins or betaherpesvirus homologues.

DNA polymerase (UL54) and polymerase accessory protein (UL44)

The HCMV polymerase and the polymerase accessory protein are among the most well-characterized HCMV proteins. Contrary to many herpesvirus proteins, the polymerase and its accessory protein appear to be easily purified as recombinant proteins and retain native function in in vitro assays. Using purified UL54 from insect cells, this protein was shown to synthesize DNA from a variety of templates. In addition, the polymerase accessory protein, the gene product of UL44, stimulated DNA polymerase activity in a template dependent manner (Ertl and Powell, 1992). In infected cells the HCMV DNA polymerase associates with the UL44 gene product (Ertl and Powell, 1992). UL54 along with UL44 were efficiently expressed in an in vitro-coupled transcription/translation reticulocyte lysate system and the activity of the enzymes was comparable to what was observed from the native infected cell purified proteins (Cihlar *et al.*, 1997). This method established a simple protocol for the expression and purification of the recombinant proteins. Homologous domains of ppUL54, with significantly conserved sequences and similar enzymatic properties in HSV and other herpesviruses have been identified (Coen, 1996). The expression of ppUL54 is controlled by multiple regulatory elements during viral infection. ppUL54 is resistant to high salt concentrations and is sensitive to particular deoxyribonucleoside and pyrophosphate analogue antiviral agents (Freitas *et al.*, 1985; Huang, 1975; Wahren *et al.*, 1985). Proteins expressed from both UL112–113 and IRS1/TRS1 loci in association with the major immediate–early (MIE) proteins, were implicated as *trans*activators of UL54 expression (Kerry *et al.*, 1996, 1997). These studies implicated cellular factors as one of the major factors involved in control of UL54 expression. Since UL54, along with the product of another conserved gene, UL97 (viral protein kinase), are targets of many antiviral compounds, many studies have focused on the genetic changes occurring in these genes in drug-resistant HCMV isolates. Several of these studies have shown that the main mechanism conferring drug resistance is through point mutations within the DNA polymerase gene locus (Cihlar *et al.*, 1998 a,b; Eckle *et al.*, 2000; Eizuru, 1998; Mousavi-Jazi *et al.*, 2001). One drug in particular, foscarnet (PFA), induced specific point mutations in a conserved region of the polymerase gene likely to be associated with pyrophospate release (Mousavi-Jazi *et al.*, 2003). Studies involving the use of siRNA demonstrated that UL54 inhibition by this method can be as efficient as conventional drug therapies (Wiebusch *et al.*, 2004).

The polymerase accessory protein, ppUL44 (originally designated ICP36) (Mocarski *et al.*, 1985), is an abundant, nuclear phosphorylated DNA-binding protein with an approximate molecular weight of 52 kDa that partitions into viral replication compartments (Anders and McCue, 1996; Gibson, 1983, 1984; Gibson *et al.*, 1981; Ho, 1991; Mocarski *et al.*, 1985). UL44 protein can be detected in infected cells during the early phase of viral infection and accumulates in the nuclei of infected cells until, and throughout, the late phase (Ho, 1991). UL44 was shown to be a substrate for UL97 protein kinase demonstrating that the phosphorylation of UL44 may be controlled by both viral encoded and cellular kinases (Krosky *et al.*, 2003). In addition, the requirement for UL44 in the transient replication assay, studies using the expression of antisense RNA to the UL44 transcript also demonstrated UL44 as being an essential protein in the context of the viral genome in virus infected cells (Ripalti *et al.*, 1995). It was shown that transactivation of the UL44 promoter depends on a gene product(s) encoded by the TRS1 segment of the genome in conjunction with IE1 and IE2 immediate–early proteins (Stasiak and Mocarski, 1992).

Because UL54 and UL44 represent significant drug targets recent studies have focused on the interaction of these two proteins. In vitro analysis determined that the C-terminal end of UL54 was essential for interaction with UL44. A small peptide, corresponding to residues 1161–1242 of UL54, effectively interfered with the interaction of the two proteins (Loregian *et al.*, 2003). Further studies indicated that specific point mutations within the UL54 protein in amino acid residues Leu1227 and Phe1231, were

sufficient to impede the interaction of UL54 with UL44, as well as affecting the ability of the enzyme complex to synthesis long chain DNA (Loregian *et al.*, 2004).

Single-stranded DNA binding protein (UL57)

The single-stranded DNA (ssDNA) binding protein has similar size and biochemical properties to the HSV-1 single-stranded DNA binding protein encoded by the UL29 gene (Anders and Gibson, 1988; Anders *et al.*, 1986, 1987; Kemble *et al.*, 1987). The HSV-1 ssDNA binding activity is essential for viral DNA replication (Lee and Knipe, 1985; Ruyechan, 1983) and exhibits a helix-destabilizing activity but can also catalyze the renaturation of complementary single strands of DNA (Dutch and Lehman, 1993). Recent studies indicate that this protein exhibits RNA binding and R-loop formation in addition to functioning as a recombinase (Boehmer, 2004; Reuven *et al.*, 2004). HSV ssDNA binding protein interacts with several other viral DNA replication proteins including DNA polymerase and the OBP, and binding may stimulate the activities of DNA polymerase, OBP helicase, and helicase–primase (Boehmer *et al.*, 1993; Boehmer and Lehman, 1993a,b; Chiou *et al.*, 1985; Falkenberg *et al.*, 1997; Hamatake *et al.*, 1997; Hernandez and Lehman, 1990; Tanguy LeGac *et al.*, 1998).

HCMV ssDNA binding protein is encoded by UL57 as a 140 kDa protein that localizes to intranuclear DNA replication compartments by 48 h post-infection and accumulates in characteristic prereplicative sites when DNA synthesis is inhibited by treatment with drugs that inhibit viral DNA synthesis (Anders *et al.*, 1987; Penfold and Mocarski, 1997).

UL84

The requirement for the core replication proteins: polymerase, polymerase processivity factor, trimeric helicase-primase and ssDNA binding protein is common among all herpesviruses. One additional protein, the UL84 gene product (ppUL84) is necessary for replication. ppUL84 is a 586aa polypeptide present in infected cells as early as 2.5 hpi (He *et al.*, 1992) and is a nuclear-localizing phosphoprotein. UL84 colocalizes with UL44 and IE2 (580 aa) in the nucleus and is a component of viral replication compartments in infected and transfected cells (Xu *et al.*, 2002). UL84 stably interacts with IE2 in infected cells (Samaniego *et al.*, 1994; Spector and Tevethia, 1994) and overexpression of UL84 decreases IE2-mediated transient transactivation (Gebert *et al.*, 1997). ppUL84 nuclear localization can either use a signal similar to the SV40 T antigen nuclear localization signal (Xu *et al.*, 2002) or a non-conventional importin alpha protein-dependent process (Lischka *et al.*, 2003). ppUL84 probably dimerizes (Colletti *et al.*, 2004). The self-interaction domain is localized to a highly charged region of the protein. UL84 is the only HCMV function needed to facilitate replication of HCMV *ori*Lyt by the six Epstein–Barr virus replication-fork proteins in Vero cells (Sarisky and Hayward, 1996). Although there were different HCMV-encoded factors required in human fibroblasts versus Vero cells, UL84 was the only auxiliary component that could not be omitted (Sarisky and Hayward, 1996). Other laboratories have observed cell type dependent requirements for HCMV replication proteins, indicating that replication may proceed by different mechanisms depending on cellular factors present (Reid *et al.*, 2003). The UL84 ORF is essential for virus growth as evidenced by the generation of recombinant viruses with insertions or replacement in UL84 (Dunn *et al.*, 2003; Xu *et al.*, 2004). Interestingly, all kinetic classes of viral transcripts are made in cells transfected with recombinant UL84 deficient viral bacmid DNA (Xu *et al.*, 2004), despite the fact that UL84-defective BACs fail to accumulate any viral DNA.

Recent evidence showed that ppUL84 acts in concert with IE2-p86 to activate a *cis*-acting element within *ori*Lyt suggesting that a ppUL84-IE2-p86 interaction may supply a "trigger" for viral DNA synthesis. Therefore, ppUL84 appears to have a dual function in HCMV DNA replication. The first is either as a coactivator of transcription along with IE2 or a repressor of IE2-mediated transcriptional activation. Activation of a promoter within *ori*Lyt may provide an essential signal for initiation of DNA synthesis. The second function may involve an as yet undefined enzymatic activity such as a helicase. This is based on the observation that the amino acid sequence of UL84 has a significant homology to the DExD/H box family of RNA helicases. Consistent with this possibility, HCMV UL84 shows UTPase activity (Colletti *et al.*, 2005). These proteins may act as either coactivators or repressors of transcription in a promoter-specific manner (Wilson *et al.*, 2004). New electromobility shift assay (EMSA) data from the Pari laboratory show that UL84 can interact with both DNA and RNA in vitro. This interaction appears to be non-specific but a higher affinity for RNA sequences that form a stem-loop suggests that UL84 may bind to these structures within *ori*Lyt. Moreover, chromatin immunoprecipitation (ChIP) assays using either infected-cell DNA or DNA contained within purified virus showed that UL84 is associated with the HCMV genome. UL84 binding was localized to three distinct regions within *ori*Lyt.

HCMV UL84 homologues are found in all betaherpesviruses (Chapter 15) but have highest DNA sequence similarity in closely related chimpanzee or baboon CMV (Davison *et al.*, 2003). This is in contrast to UL84 homologues found within MCMV and GPCMV, or in the roseolaviruses where the UL84 ORF retains little similarity to

the HCMV counterpart. This lack of sequence homology also appears to translate to a functional distinction between primate CMVs and other species since it was demonstrated that the UL84 ORF is non-essential for virus growth in both MCMV and GPCMV (Morello *et al.*, 2002) (M. Schleiss and A. McGregor, personal communication).

Origin binding proteins in roseoloviruses

Initiator proteins for HHV6 and HHV7 appear to have close homology to OBP of HSV-1. These proteins, the gene product of U73 for both HHV6 and HHV7, each interact with two sites within the *ori*Lyt for both viruses. These sequences are equivalent to Box I and Box II of HSV-1 *ori*Lyt, flank AT-rich sequences and exhibit other similar sequence characteristics. Other DNA replication proteins within HHV6 and HHV7 have been assumed based on nucleotide sequence homology to the HSV-1 and HCMV functions. They include: U38 (DNA polymerase), U27 (polymerase accessory protein), U43, U74 and U77 (helicase–primase complex) and U41 (ssDNA binding protein).

Immediate–early protein IE2–580aa

As is summarized in Chapter 17, IE2 encodes an 86-kDa nuclear immediate–early phosphoprotein that is essential for lytic replication in tissue culture (Heider *et al.*, 2002; Marchini *et al.*, 2001). IE2-p86 is thought to be the major transcription-activating protein of HCMV and is also responsible for negative autoregulation of the major immediate–early promoter (MIEP) (Mocarski, 2001). IE2 has been implicated in multiple protein–protein interactions including the formation of dimers, association with ppUL84 and several transcription factors, for example CREB, CBP, SP-1, and c-Jun (Chiou *et al.*, 1993; Furnari *et al.*, 1993; Lang *et al.*, 1995; Lukac *et al.*, 1994; Schwartz *et al.*, 1996; Scully *et al.*, 1995; Wara-aswapati *et al.*, 1999).

The role of IE2 with respect to DNA replication is less well understood. It has been shown that IE2-p86 interacts with ppUL84 to activate a responsive promoter within *ori*Lyt (Xu *et al.*, 2004). However, the requirement for IE2 in origin-dependent DNA replication is apparently cell type dependent. The *ori*Lyt promoter is constitutively active in Vero cells, thereby enabling amplification of *ori*Lyt in the absence of IE2 (Xu *et al.*, 2004).

UL36–38

Although these genes were identified as being required for DNA replication (Pari and Anders, 1993), they appear to play an auxiliary role (Sarisky and Hayward, 1996). In addition, in a human fibroblast cell line immortalized with the catalytic subunit for telomerase (hTERT), the gene products of UL36–38 are no longer necessary for origin-dependent DNA replication (Xu *et al.*, 2004). These proteins may function to facilitate a more favorable cellular environment for transient DNA replication. The UL36–38 locus encodes at least five transcripts from three different transcriptional promoters: the UL36, UL37, UL37$_M$, UL37×1 and UL38 RNAs (Goldmacher *et al.*, 1999; Kouzarides *et al.*, 1988; Tenney and Colberg-Poley, 1991a,b; Wilkinson *et al.*, 1984). Despite being initiated at the same IE promoter, three UL37 RNAs, UL37×1, UL37, UL37$_M$, are generated by differential RNA splicing and polyadenylation and show dramatically different temporal expression during HCMV infection. The UL37×1 unspliced RNA is expressed abundantly at IE times and remains abundant until late times of infection. In contrast, the other two UL37 RNAs, which are spliced, are expressed at low abundance during IE times and encoding two UL37 glycoproteins (gpUL37 and gpUL37$_M$) that are dispensable for replication. Only pUL37×1 is required for viral replication (Reboredo *et al.*, 2004) possibly via its anti-apoptotic activity (Goldmacher *et al.*, 1999; Hayajneh *et al.*, 2001).

Betaherpesvirus replication origins

Cytomegalovirus *ori*Lyt

Several lines of evidence show that a unique HCMV origin of lytic DNA synthesis, called *ori*Lyt, lies near the middle of U$_L$, between the UL57 and UL69 ORFs. First, this region directs virus-mediated replication of plasmids in transient replication assays (Anders and Punturieri, 1991). Such assays allowed the mapping of functional replicator sequences (Anders *et al.*, 1992; Masse *et al.*, 1992; Zhu *et al.*, 1998); see below). Second, evidence from an inhibitor study indicates that DNA synthesis initiates in or near this region of the virus genome during productive infection of permissive cells (Hamzeh *et al.*, 1990). Third, although poorly conserved at the nucleotide sequence level, the position of *ori*Lyt next to the single-stranded DNA binding protein gene is conserved in all other cytomegaloviruses studied to date, and the corresponding regions confer replication competence in the transient assay (Masse *et al.*, 1997; Vink *et al.*, 1997). Finally, in contrast to the lytic replicators of many other herpesviruses, cytomegalovirus *ori*Lyt appears to be single-copy, because it is not duplicated in the genomes of either laboratory or clinical isolates, and recombinant HCMV BACs deleted for *ori*Lyt fail to produce virus or replicate their genomes (Dunn *et al.*, 2003; D. G. Anders, unpublished results; Borst and Messerle, 2005). This is consistent with previous findings that no other

segment of the viral genome has replicator activity in the transient assay. Therefore, *ori*Lyt is thought to be essential for lytic replication in the host. Because it is essential and may play an important regulatory role in the virus–host relationship, we provide a complete summary of work-to-date.

Cytomegalovirus *ori*Lyt structure

Cytomegalovirus *ori*Lyt is distinguished from most other herpesvirus replicators, including those of the roseoloviruses, in that it is large and complex. Some structural features are shared with gammaherpesvirus *ori*Lyt sequences. Deletions to define the external borders established a minimal *ori*Lyt region containing essential elements, and showed that sequences spanning more than 2.5 kbp can contribute to replicator activity in transient assays (Anders *et al.*, 1992; Masse *et al.*, 1992). Kanamycin cassette insertion studies defined a similar core region, roughly nt 91 750 to 93 300, within which the insertions either eliminated or greatly impaired replicator function (Zhu *et al.*, 1998). This minimal oriLyt core region does not support efficient replicator activity, at least in transient assays, in the absence of left or right flanking sequences. The flanking sequences independently contribute to *ori*Lyt activity with relaxed position dependence, in that insertions do not significantly compromise activation of the *ori*Lyt core, and therefore they have been referred to as "auxiliary" sequences.

Two notable features characterize the cytomegalovirus *ori*Lyt region. First, it contains a high density of direct and inverted repeats (Masse *et al.*, 1992). Several of these appear virus specific, including multiple copies of a 10-bp motif similar to the known binding site for the UL34 protein clustered in the left auxiliary region, and 13- (also known as FspI/SphI) and 19-bp directly repeated sequences within the core region. Reiterated sequences similar to the 10-bp and 13-bp motifs are present at corresponding positions in other cytomegaloviruses. Two large, but imperfect inverted repeats are present on the right side of the *ori*Lyt core region; similar structures are present at the corresponding positions of all known CMV *ori*Lyt sequences. Interestingly, these can be variably reiterated in laboratory strains (Prichard *et al.*, 1998). Other repeated elements include a variety of consensus host transcription factor recognition sequences, most notably clusters of potential Sp1 and CREB sites. Which of these reiterated motifs contribute to replicator activity, how and under what circumstances, remains to be determined. A second characteristic feature of CMV *ori*Lyt is its overall base composition asymmetry. Most of the *ori*Lyt region approximates the overall GC content of the viral genome, but AT-rich segments lie on the left side, and a very GC-rich region on the right. The imperfect inverted repeats lie in the GC-rich region. EBV *ori*Lyt shows a similar organization. The roles of AT-rich and GC-rich segments are not understood, but the AT-rich segment in the leftward minimal activating sequence contributes to activation of the core replicator in transient assays (Kiehl *et al.*, 2003).

A series of overlapping 200-bp deletions spanning the *ori*Lyt core region were made and tested for activity in the transient replication assay to search for essential elements (Zhu *et al.*, 1998). All except one of the core deletions reduced replicator activity from about 20- to greater than 100-fold, suggesting that elements contributing to replicator activity are scattered throughout the core region, or that spatial relationships of distant elements are important. Two regions critical for replicator activity were identified by this approach. One of these, extending from nt 92 400 to 92 573, includes the only known individually essential element, a pyrimidine (Y)-rich tract followed by a purine (R)-rich tract of nucleotides, referred to as a "Y-block." Y·R elements are present in all other CMV *ori*Lyt sequences as well as in EBV *ori*Lyt, but are absent from origins that characterize HHV-6, HHV-7 and alphaherpesviruses that interact with an origin binding protein (Huang *et al.*, 1996). Nucleotide substitutions in this element eliminate replicator activity in the transient assay. The other essential segment spans nt 92 887 to 93 145, and contains the cluster of Sp1 consensus sites and overlaps the vRNA-2 region. A 405 bp deletion starting at starting at 92 574, which was less than threefold reduced in activity relative to the wild type in the transient replication assay, identified the only dispensable sequence in the *ori*Lyt core region. Lastly, because the core region alone has minimal activity in the transient assay, auxiliary sequences probably play an important role in activating *ori*Lyt. A minimal *ori*Lyt activating sequence overlapping the UL57 promoter was defined by reconstitution, providing a model to study how auxiliary sequences enhance core replicator function (Kiehl *et al.*, 2003).

Finally, there are two important caveats to conclusions regarding functional elements of CMV *ori*Lyt. First, they were drawn on the basis of transient assays. Specific sequence requirements for *ori*Lyt function in the context of the virus genome have yet to be investigated. Second, most of this work was done in permissive fibroblasts; comparative studies in other cell types important in the infected host, in which different sets of cellular regulatory proteins predominate, may reveal distinct sequence requirements. Indeed, the HCMV *ori*Lyt bidirectional promoter $ori\text{Lyt}_{PM}$ is constitutively active in Vero cells, but requires both IE2 and UL84 for activity in fibroblasts (Xu *et al.*, 2004).

*ori*Lyt open reading frames and transcripts

The HCMV *ori*Lyt region includes predicted ORFs UL58-UL61, and a newly described ORF 3 lies at the right boundary (Murphy *et al.*, 2003a). In addition, the *ori*Lyt region is actively transcribed and several transcripts have been characterized. However, the predicted proteins are not conserved in other cytomegaloviruses including chimpanzee CMV and moreover, with the exceptions of UL59 and to a lesser extent ORF3, the *ori*Lyt ORFs are not conserved amongst clinical isolates, suggesting that they may not represent authentic protein-coding genes (Murphy *et al.*, 2003b; see Chapter 14).

The UL59 ORF is interesting in that it is absent in other other CMV genomes sequenced to date, and appears to have been recently acquired as an insertion between the organizationally conserved *ori*Lyt core and leftward auxiliary regions. It is better conserved among clinical isolates than the other potential *ori*Lyt coding sequences, and a corresponding late transcript of 0.4–0.7 kb is made. However, it is not known whether the predicted protein is expressed. The segment upstream of UL59, extending about 900 bp to Y·R element, is rich in consensus transcription factor binding site sequences, and contains an apparently bidirectional promoter, *ori*Lyt$_{PM}$, that is regulated by IE2 and UL84 (Xu *et al.*, 2004). Transcripts spanning UL59 initiate from several sites in this region. Deletion of the UL59 ORF does not affect HCMV growth in fibroblasts, but tests of growth in other cell types have not been reported (Dunn *et al.*, 2003).

Recombinant HCMV BACs carrying an insertion in either the UL60 or UL61 ORF or a deletion of the UL60 ORF failed to replicate, but these mutations would be predicted on the basis of previous findings to inactivate *ori*Lyt, and therefore more subtle mutations would be needed to determine whether these ORFs are essential (Dunn *et al.*, 2003; Yu *et al.*, 2003). A second, late transcript of around 6.5 kb that has the same orientation and is 3 co-terminal with the UL59 transcript crosses *ori*Lyt from right-to-left, but the complete structure of that transcript is not known.

Three other *ori*Lyt RNA species have been described. The first, called the small replicator transcript, is a roughly 250 nt early, non-polyadenylated species with a single 5′ end and a variable 3′ end that overlaps the essential "Y-block" element (Huang *et al.*, 1996). It lies within the UL60 ORF. An upstream promoter for the small replicator transcript is active in transient assays, but studies to confirm this in the genome context have yet to be done. Two other RNAs, called virus-associated or "vRNAs," were found to be covalently associated with progeny DNA at least partly in RNA–DNA hybrid form, and present in virions (Prichard *et al.*, 1998). vRNA-1 localizes between nt 93 799 and 94 631, and vRNA-2 between nt 92 636 and 93 513. Nothing is known about how these transcripts are expressed or how they function. The apparently non-coding nature of the small replicator transcript and the vRNAs, the timing of their expression, their unusual properties and their association with genetically defined segments essential for *ori*Lyt activity have led to suggestions that they may participate in initiation of DNA synthesis.

The mechanism of initiation

Initiation of CMV DNA synthesis is not understood. Based on the information available from a variety of DNA replication systems that have been studied, initiation can be predicted to require several steps, including (i) an initial strand separation followed by unwinding, and (ii) assembly of replication fork proteins to form an active complex at the site of initiation. In SV40, T-antigen binds to specific origin sequences to form a complex that, in cooperation with certain structural features of the origin, untwists and subsequently, with an intrinsic helicase activity, unwinds the origin duplex. In addition, it directs polymerase alpha/primase association. The alphaherpesviruses origin-binding protein (e.g., HSV-1 UL9), as well as the homologous HHV-6 and HHV-7 proteins, are thought to play an analogous role, using an essential intrinsic helicase activity to provide the initial unwinding in cooperation with the single-strand DNA binding protein. However, as noted above, the cytomegaloviruses lack a UL9 homologue. Therefore, it is not clear how initial unwinding occurs or how the replication fork proteins may be directed to load. One frequently discussed possibility is that RNA–DNA hybrids formed during transcription may produce open regions that could be used to load the helicase–primase complex. This suggestion is supported by the synthesis of candidate RNAs and the observation of residual RNA-DNA hybrids, the vRNAs, in replicated viral DNA at sites critical to *ori*Lyt activity. This could explain, at least in part, the complexity of CMV *ori*Lyt, as this mechanism requires not only the transcript itself, but also control regions for transcription and elements to direct assembly to the replication fork complex. However, there are many other possibilities including co-opting the cellular initiation machinery, as EBV does for latent phase DNA synthesis. Initiation remains one of the most interesting areas for investigation of CMV DNA synthesis.

Roseolovirus replication origins

Human herpesviruses 6 and 7 lytic-phase replicators were identified using a transient replication assay (Dewhurst

et al., 1993; van Loon *et al.*, 1997). The roseolovirus lytic origins are located adjacent to the single-stranded DNA binding protein gene, U41, a position that is similar to CMV *ori*Lyt; however, the structure of roseolovirus *ori*Lyt exhibits similarity to that of the alphaherpesviruses. As is the case for cytomegalovirus, HHV-6 and HHV-7 *ori*Lyt sequences are thought to be unique, although this has not been fully tested. The HHV-6 *ori*Lyt region is unusually AT rich, and includes a large, imperfect directly repeated (IDR) sequence. In contrast, HHV-7 *ori*Lyt is more compact and is missing the IDR region and adjacent sequences that contribute to HHV-6 replicator activity. Both are smaller and less structurally complex than cytomegalovirus *ori*Lyt, and include comparatively fewer potential transcription factor recognition sequences. Plasmids carrying HHV-6 *ori*Lyt were replicated following introduction into either HHV-6- or HHV-7-infected cells, although HHV-7 *ori*Lyt was only replicated in HHV-7-infected cells (van Loon *et al.*, 1997).

In striking contrast to HCMV *ori*Lyt, the roseolovirus replicators are characterized by the presence of at least two binding sites for a virus-coded protein, U73, homologous to the alphaherpesvirus OBP. As noted above (see Chapter 15), OBP homologues are not found in the cytomegaloviruses. As detailed earlier, the roseolovirus OBP homologues bind specifically to defined oriLyt sites in vitro (Inoue *et al.*, 1994; Inoue and Pellett, 1995; Krug *et al.*, 2001), and mutation of either site, or of the spacer region separating them, inactivates the replicator when tested in the transient assay (Dewhurst *et al.*, 1994; Dykes *et al.*, 1997). Therefore, OBP binding to the *ori*Lyt sites is probably essential for lytic viral replication, and mechanistically the roseolovirus replicators appear to resemble alphaherpesvirus counterparts rather than those of the cytomegaloviruses. Nevertheless, it is noteworthy that, although the HHV-6 minimal replicator spans only about 400 bp including the OBP sites, flanking sequences boost replicator activity in the transient assay suggesting that they may play an important role in regulating activity in the context of the virus genome. The minimal efficient origin includes the IDR region, which greatly enhances activity by an unknown mechanism (Dewhurst *et al.*, 1993). The IDR spans the most A+T rich segment, which may serve as a "DNA unwinding element." Comparison of the HHV-6A and B strain *ori*Lyt sequences revealed that they are more than 95% identical over the minimal essential region including the OBP binding sites, but considerably divergent in the auxiliary flanking regions, particularly beyond the IDR sequences. HHV-6A strains contain three copies of the imperfect direct repeat, whereas HHV-6B carries two copies. Interestingly, a sequence similar to the experimentally defined HHV-6 minimal efficient origin was reiterated in some cultured lineages of HHV6B, presumably by homologous recombination, and the reiterated *ori*Lyt enhanced replicator activity in the transient assay (Dewhurst *et al.*, 1994; Stamey *et al.*, 1995). This is reminiscent of the reiterations seen in HCMV *ori*Lyt laboratory strains. Specific elements within the auxiliary regions contributing to replicator activity have not been identified and, aside from the OBP binding sequences, little is known about the functional elements regulating the roseolovirus replicator.

Latent phase replication

EBV and other herpesviruses that establish latency in replicating cell types have a separate origin (*ori*P) that is responsible for maintenance and replication of the viral genome during latency. However, no latent phase origin has been identified in the cytomegalovirus genome. Indeed, a latent origin may not be required if the primary sites of latency are in non-dividing cells, particularly if the latency reservoir is replenished by cycles of reactivation as the latency hosts are stimulated to differentiate and divide. Nevertheless, little is known about the molecular aspects of betaherpesvirus latency, and the existence of a latency maintenance element cannot be excluded.

Late gene expression

Replication of the viral genome provides an essential activating event for the expression of high levels of the betaherpesvirus late genes. It is widely understood that late genes encode for proteins required for virus assembly and egress. These genes can be divided into two broad classes. The gamma 1 or leaky-late class are expressed at very low levels at early times after infection and are dramatically upregulated at late times. In contrast, the gamma 2 or "true" late genes are expressed exclusively after and are dependent upon viral DNA replication. The mechanism that restricts late gene activation to after DNA replication has not yet been determined, although at least two general possibilities have been considered. First, the restriction may be related to DNA structure, or possibly DNA modifications that are altered immediately post replication. Alternatively, binding of viral or cellular proteins to repressor elements within late promoters may inhibit late gene expression, with subsequent displacement or titration of the repressors being accomplished by DNA replication.

Our understanding of late gene regulation in the betaherpesviruses lags far behind that of early gene regulation (Chapter 17). Only a handful of late gene promoters have been analyzed in detail. One of the primary reasons for this has been the difficulty in recapitulating the appropriate kinetic regulation of late genes in transient

transfection/superinfection assays. Initial studies of betaherpesviruses late promoters in this type of assay system revealed that upon superinfection, promoter activity could be detected at early times after infection (Depto and Stenberg, 1992; Rudolph *et al.*, 1990). Thus, it was assumed that transient assays would not provide complete information regarding the necessary promoter components required to restrict viral gene expression to late times, requiring the assessment of these promoters in the context of the viral genome. In this respect, the recent development of the bacterial artificial chromosome (BAC) system to rapidly generate viral mutants will likely facilitate the analysis of late gene regulation.

However, even though a limited subset of late genes have been analyzed to date, some general features associated with their regulation have been identified. Typically, late promoters seem to require a TATA element but no further upstream sequences for transcriptional activation (Depto and Stenberg, 1989, 1992; Kohler *et al.*, 1994; McWatters *et al.*, 2002; Wing *et al.*, 1998). Similar findings have been observed for late gene promoters of herpes simplex virus, where the TATA box and downstream elements are sufficient to direct late gene activation (Flanagan *et al.*, 1991; Homa *et al.*, 1986; Weir, 2001). This is in contrast to the betaherpesviruses early promoters, where specific upstream sequence elements capable of binding to cellular transcription factors are critical for promoter activation. The most extensive studies on late gene regulation published to date have examined the human cytomegalovirus UL99 gene encoding the virion tegument protein pp28 (Depto and Stenberg, 1992; Kerry *et al.*, 1997; Kohler *et al.*, 1994). This gene, which is expressed as a gamma 2 late gene, requires only sequences from -40 relative to the cap site to restrict expression to late times (Kohler *et al.*, 1994). In order to accurately replicate appropriate promoter kinetics, these studies were performed by introducing the promoter construct regulating the CAT reporter gene into an alternate, transcriptionally inert site in the HCMV genome. This type of study led to the hypothesis that late promoters are relatively simple promoters consisting primarily of a TATA element and downstream sequences and lacking a requirement for multiple transcriptional regulatory elements.

Despite some limitations, transient assays have yielded important information about the minimal promoters of a number of late genes. (Depto and Stenberg, 1989; McWatters *et al.*, 2002; Wing *et al.*, 1998). For example, in the analysis of the HCMV UL75 promoter, which controls expression of the envelope glycoprotein gH, sequences from −38 to +15 relative to the cap site were sufficient to activate this promoter (McWatters *et al.*, 2002). However, although additional regions of the promoter were not essential for activation, closer examination of those sequences revealed a much more complex mode of regulation (McWatters *et al.*, 2002). Specifically, sequences downstream of the UL75 cap site appeared to function as a dominant regulatory element. In the absence of this region, both activation and repressor elements were identified in the upstream sequences. (McWatters *et al.*, 2002). Similarly, analysis of the HCMV UL94 late promoter identified a dominant regulatory element downstream of the cap site that functioned as a derepressor (Wing *et al.*, 1998). In the absence of this downstream element, deletion of the upstream sequences enhanced promoter activity, suggesting the presence of an upstream negative regulatory element. This negative regulatory element contained two p53 binding sites that were important, although not sufficient for the repressive effects (Wing *et al.*, 1998). These studies suggest that the regulation of late gene expression may be more complex than previously thought, with additional sequences allowing the binding of viral and/or cellular proteins that may influence the regulation of late promoters. However, it remains to be determined if these elements truly play a role in late gene regulation in the context of a normal viral infection, or if these are artifacts resulting from the assay system.

Studies suggesting a more complex model for late promoter activation are intriguing, given that a number of HCMV late promoters have been identified in the midst of both immediate early and early gene regions (Leach and Mocarski, 1989; Leatham *et al.*, 1991; Puchtler and Stamminger, 1991). In fact, in some cases, specific late transcripts with alternate TATA elements and start sites have been identified within HCMV early promoters (Jones and Muzithras, 1991; Leach and Mocarski, 1989). Thus it might be expected that adjacent regulatory elements or enhancers within the promoter context could influence the activation or kinetics of expression of such late transcriptional units. In this context, it is important to note that analyses of late promoter regulation in the context of the viral genome have been performed in a transcriptionally inert region (Kohler *et al.*, 1994), and the association of repressors and activators may be critical for restricting viral late gene expression within their natural genomic context. Thus, a more complex model of late promoter regulation emerges, with a possible role for multiple repressor and activator proteins influencing the kinetics of gene expression.

Some efforts have been made to identify specific regulatory proteins that are involved in the activation of HCMV late promoters. With respect to cellular transcription factors, analysis of the UL75 promoter indicated a role for a cellular PEA3-related protein in transcriptional activation (McWatters *et al.*, 2002). Inversely, a role for p53 in transcriptional repression of the UL94 promoter has been identified (Wing *et al.*, 1998). Some recent studies suggest

that the NF-κB transcription factor may also be involved in activating viral late gene expression (DeMeritt *et al.*, 2006). Other studies have identified sequence elements involved in late promoter activation, although the specific proteins required for activation remain to be identified (Depto and Stenberg, 1989, 1992). Intriguingly, a more global analysis of the putative promoter regions of HCMV late genes revealed a palindromic GC-rich sequence (CCGCGGGCGCGG) in the promoters of 17% of viral late genes (Chambers *et al.*, 1999). The significance of this sequence element in late gene regulation remains to be determined.

In addition to cellular proteins, there is evidence for the role of viral factors in the activation of late promoters. The fact that late kinetics are not conserved when the promoters are relieved of the constraints of the viral genome suggests that the viral factors required for activation are present at early times of infection. Indeed, the HCMV immediate–early proteins can activate some late promoters, although to minimal extents (Depto and Stenberg, 1989, 1992; Wu *et al.*, 2001). Other HCMV proteins, such as trs1 may also play a role in activating late promoters (Romanowski and Shenk, 1997; Stasiak and Mocarski, 1992). The finding that the IE proteins of HCMV may be involved in regulating viral late gene expression is interesting in the light of recent studies examining the role of IE2 during viral infection (Sanchez *et al.*, 2002; White *et al.*, 2004). Deletion of IE2 sequences from amino acids 136 to 290 resulted in reduced amounts of IE2 in infected cells but had minimal impact on viral DNA replication (Sanchez *et al.*, 2002). Interestingly, two viral late proteins, pp65 and pp28 were found to be expressed at reduced levels. Likewise, UL83 mRNA levels that encode the pp65 gamma 1 late protein were reduced (Sanchez *et al.*, 2002). Small internal deletions in IE2 from amino acids 356–359, 427–435, and 505–511 and one substitution mutant of IE2 were also assessed using the same strategy (White *et al.*, 2004). The deletion of amino acids 356–359 and the substitution mutant were both defective in late gene expression. In contrast, deletion of amino acids 427–435 and 505–511 resulted in an IE2 protein that did not activate early gene expression, but expressed certain gamma 1 and gamma 2 genes at early times after infection. Sequence analysis located a potential IE2-binding site within the promoter region of one of these genes, suggesting that IE2 may be involved in transcriptional repression of late genes (White *et al.*, 2004). These findings suggest that IE2 plays a hitherto unrecognized role in directly regulating late gene expression in both a positive and a negative manner.

A role for IE2 in late promoter regulation would appear to be in conflict with the kinetics of expression of this protein. One possibility is that IE2 is differentially modified at late times after viral infection. IE2 can be modified by both phosphorylation and sumoylation, and differential modification through the course of infection could influence the ability of IE2 to regulate gene expression (Heider *et al.*, 2002; Hofmann *et al.*, 2000; Lee *et al.*, 2003). Alternatively, an IE2-related protein, p40 corresponding to amino acids 242–580 of IE2 (Plachter *et al.*, 1993) has been characterized that is expressed via an alternate promoter at late times. This protein has been demonstrated to function as a transactivator, but its specific role during infection has not been defined (Jenkins *et al.*, 1994). The possibility exists that the p40 protein is involved in the activation of late promoters. Indeed, the IE2 deletion from amino acids 136 to 290 that exhibits defects in late gene expression removes the start codon for the p40 late gene product from the IE2 region (Sanchez *et al.*, 2002). Thus, IE2 or IE2-related proteins may play a differential role in transcriptional activation of viral promoters at multiple stages of viral infection. Recent studies have identified a gammaherpesvirus gene, ORF18 that is essential for late gene expression (Arumugaswami *et al.*, 2006). While a direct role for the betaherpesvirus homolog of ORF18, UL79 in late gene activation has yet to be assessed, it is highly likely that additional viral proteins will be found to be important for the regulation of late promoters.

To date, there has been only one study addressing the direct role of DNA replication in stimulating late gene transcription. In this analysis, the HCMV TRL7 gene that is expressed with gamma 1 kinetics was assessed in the context of a plasmid containing the HCMV *ori*Lyt region (Wade and Spector, 1994). This approach restored the dependence on DNA replication for high levels of the TRL7 promoter-directed transcript in transient assays. While the plasmid itself did replicate upon superinfection with HCMV, the level of replication appeared to be insufficient to explain the increased transcription rates. In addition, enhanced transcription of the gamma 1 gene at late times did not appear to be related to methylation differences, or dependent on specific upstream promoter elements. One intriguing possibility discussed by the authors is that factors required for replication may play a dual role in both replication and transcriptional activation, with replication providing a mechanism for bringing such factors into proximity of the promoter, and thereby providing for enhanced transcription, based on a model originally identified in the bacteriophage T4 (Herendeen *et al.*, 1989, 1992).

In addition to transcriptional events, there is evidence that post-transcriptional regulation may play a critical role in controlling the appearance of viral late gene products. Analysis of the HCMV UL99 5' untranslated region revealed that these sequences influenced translational regulation, via a putative stem–loop structure (Kerry *et al.*, 1997). While

the mechanism of this regulation has not yet been characterized, recent analysis of IE2 mutants revealed that this protein may be involved, suggesting that the influence of IE2 on late gene expression is at multiple levels (White *et al.*, 2004). Post-transcriptional regulation has also been identified as a potential influence on the expression of late genes in the other betaherpesviruses, HHV-6 and -7. For these two viruses, specific splice variants that are restricted to the late phase of viral infection have been identified (Menegazzi *et al.*, 1999; Mirandola *et al.*, 1998). Thus, post-transcriptional control of gene expression at the late stages of viral infection may be a common mechanism in the betaherpesviruses to control the function and time of appearance of viral proteins. Post-transcriptional mechanisms also play an important role in the regulation of late gene expression during herpes simplex virus infection (McCormick *et al.*, 1999; Perkins *et al.*, 2003), suggesting that this may represent a common theme in the control of late gene expression throughout the herpesviruses.

In summary, although minimal sequences may be sufficient to restrict expression to late times in a transcriptionally inactive environment, a more accurate picture of the complexity of betaherpesvirus late gene regulation is now emerging. A role for viral and cellular transcriptional regulators, both positive and negative, has been identified, although definitive studies to truly assess late promoter requirements in a natural genomic environment remain to be performed. In addition, post-transcriptional events likely play a critical role in controlling the time of appearance of viral late proteins. Such strict control over the expression of late proteins is likely to be key for the appropriate assembly and egress of new virion particles.

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Maturation and egress

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Introduction

The assembly of betaherpesviruses, specifically cytomegaloviruses, is a topic of considerable interest to virologists and structural biologists. These viruses are among the largest and most complex animal viruses and encode a large number of proteins. Some clinical isolates of human cytomegalovirus (HCMV) have been predicted to contain as many as 250 ORFs (Chee *et al.*, 1990; Murphy *et al.*, 2003), while other authors have suggested that the coding capacity of HCMV may actually be on the order of 165 ORFs (see Chapter 14). Although the number of virus-encoded proteins that are incorporated into the infectious particle is unknown, estimates from several laboratories suggest that it could approach 100 proteins (Varnum *et al.*, 2004). In addition, the particle also contains an unknown number of host cell proteins, some that may have functional significance in the replicative cycle of these viruses (Varnum *et al.*, 2004). Thus, the complexity of virus assembly rivals that of some cellular organelles. Furthermore, CMVs do not arrest host cell protein synthesis even at late phases of replication as do the alphaherpesviruses and therefore during their assembly can either compete with host cell protein synthetic and targeting pathways or more likely, express viral specific functions that modulate host cellular pathways to optimize viral protein synthesis and transport. Identification of these virus-specified host cell modifications together with their interactions with virion proteins will aid in the understanding of the assembly of this virus. Similar approaches have provided a reasonably detailed view of the assembly pathways of bacteriophages and eukaryotic RNA viruses, including HIV-1, and these studies have in some instances served as templates for investigations of DNA virus assembly. However, herpesviruses encode vastly more virion proteins compared to RNA viruses, and assembly takes place in both nuclear and cytoplasmic compartments. Definitive studies of capsid assembly have been carried out in alphaherpesviruses and have been extended to include in vitro, cell free assembly of the herpes simplex viral (HSV) capsid (Newcomb *et al.*, 1994). Many of the capsid proteins of CMVs and other betaherpesviruses share extensive structural and functional homology with HSV capsid proteins and cryoelectron microscopic analysis of the HCMV capsid suggests similar, but not identical, capsid structure (Butcher *et al.*, 1998; Chen *et al.*, 1999; Trus *et al.*, 1999; Zhou *et al.*, 1999, 2000). The most structurally diverse region of the herpesvirus virion appears to be the tegument which is composed of a great many betaherpesvirus- and CMV-unique proteins. Although proteins with homologous functions localized to this region of the virus can be readily identified for many different herpesviruses, only a limited number of these proteins exhibit significant structural homologies (Mocarski and Tan Courcelle, C., 2001). Despite these similarities, a large number of structural proteins appear to be unique for each subfamily of herpesviruses and in some cases, such as HCMV, several tegument proteins have no counterparts in alphaherpesviruses (Mocarski and Tan Courcelle, C., 2001). Together, these findings suggest that, although common themes likely exist for herpesvirus assembly, it is almost certain that distinct aspects of the assembly pathway of each of these viruses will be identified and these differences in the assembly of different herpes viruses could point to key features of the infectious cycle of these viruses.

Early studies of the assembly of alphaherpesviruses including HSV suggested that virion envelopment occurred at the nuclear membrane (Campadelli-Fiume *et al.*, 1991; Johnson and Spear, 1982). This concept was extended to other herpesviruses including HCMV, even though early studies utilizing electron microscopy suggested major differences between the morphogenesis of these two viruses, both within the infected cell and in the

overall appearance of extracellular virions (Smith and DeHarven, E., 1973). Subsequent studies of alphaherpesviruses provided evidence for nuclear envelopment–de-envelopment followed by envelopment at cytoplasmic membranes (Granzow *et al.*, 1997; Jones *et al.*, 1988; Whealy *et al.*, 1991). These reports provided clear evidence that perinuclear virions contained a different subset of tegument and envelope proteins than mature virions, findings that support acquisition and then loss of an immature envelope by perinuclear virions (Brack *et al.*, 1999; Granzow *et al.*, 1997, 2001; Klupp *et al.*, 2000). These results have been further supported by more recent studies of viruses with deletion mutations in genes encoding both virion envelope and tegument proteins (Brack *et al.*, 1999; Fuchs *et al.*, 2002; Kopp *et al.*, 2003). The elegant studies of pseudorabies virus (PRV) have provided definitive evidence of cytoplasmic virion envelopment and served as a guide for studies of the envelopment of β-herpesviruses (Mettenleiter, 2002). The envelopment of CMVs, specifically HCMV, also appears to take place within a cytoplasmic compartment based on studies of tegument protein trafficking and incorporation into the virion particle (Britt *et al.*, 2004; Sanchez *et al.*, 2000a; Silva *et al.*, 2003). Major gaps remain in the understanding of capsid tegumentation, capsid egress from the nucleus, and cytoplasmic assembly of the mature, infectious HCMV particle. The development of bacterial artificial chromosome (BAC) technology that allows the maintenance of HCMV as infectious clones coupled with the application of techniques of recombinant prokaryotic genetic manipulation has accelerated experimental studies in HCMV assembly (Adler *et al.*, 2003; Borst *et al.*, 1999; Messerle *et al.*, 2000; Smith and Enquist, L. W., 1999). Together with other technologies such as mass spectrometry and cryoelectron microscopy, current studies should provide more definitive understanding of this complex process. In the following review, the focus will be on the assembly of HCMV as a model for betaherpesvirus assembly because the majority of studies have been carried out in systems utilizing HCMV or the related virus, murine CMV (MCMV). When possible, studies of other betaherpesvirus will be discussed.

Assembly of the capsid

Structural studies of the HSV capsid have indicated that structural and functional protein homologues of HSV capsid proteins are present in the capsid of HCMV, HHV-6, and HHV-7 (Table 20.1). Cryoelectron microscopic analysis of the capsids of HCMV and HSV have revealed near identical structures, albeit with slight differences in the floor of the capsid, perhaps because of the larger size of the HCMV genome (Butcher *et al.*, 1998; Chen *et al.*, 1999; Trus *et al.*, 1999; Zhou *et al.*, 2000). Because of this structural conservation, it has been assumed that the assembly of the HCMV capsid as well as capsids of other β-herpesviruses follow a very similar assembly pathway as that of HSV (Steven *et al.*, 1997; Grunewald *et al.*, 2003). The size of the HCMV particle is approximately 2000 angstroms. The nucleocapsid is approximately 1300 angstroms and is of iscosahedral $T = 16$ symmetry (Butcher *et al.*, 1998; Chen *et al.*, 1999). As noted in Table 20.1, there are six identified protein components of the mature HCMV capsid present in the infectious particle (Gibson, 1996). Interestingly, the capsid proteins from both HSV and HCMV share considerable structural and biochemical characteristics and, in the case of MCP, even express cross-reactive antibody binding sites (Rudolph *et al.*, 1990).

The HCMV capsid is composed of 162 capsomeres consisting of 150 hexons and 12 pentons (Butcher *et al.*, 1998; Chen *et al.*, 1999). The most abundant protein components of the capsid is the major capsid protein (MCP, UL86) and the smallest capsid protein (SCP, UL48–49), with 960 and 900 copies respectively present in each capsid. Two copies of the minor capsid protein (MnCP, UL85; triplex dimer or TR-2) combined with a single copy of the minor capsid binding protein (MnCP-bp; triplex monomer or TR-1) UL46 form the triplexes that are located between adjacent pentons and hexons (Fig. 20.1) (Butcher *et al.*, 1998; Chen *et al.*, 1999; Gibson, 1996; Gibson *et al.*, 1996; Trus *et al.*, 1999). The capsid pentons and hexons are assembled entirely from the major capsid protein. Each hexon is decorated on each vertex with the smallest capsid protein (Yu *et al.*, 2005). The smallest capsid protein of HSV, Vp26, has been shown to be dispensable for capsid assembly in cell culture, whereas deletion of the HCMV homologue results in the loss of infectivity, presumably by preventing the assembly of infectious virions (Borst *et al.*, 2001). A cartoon depicting the assembly pathway is detailed in Fig. 20.1.

Protein interactions and capsid assembly

As noted above, evidence from transmission electron microscopy and cryo-EM, combined with the conservation of capsid protein function, suggests that the capsid assembly of HCMV and other betaherpesviruses closely follows that of HSV (Beaudet-Miller *et al.*, 1996; Gibson, 1996, Gibson *et al.*, 1996, 1990, 1993; Welch *et al.*, 1991a,b; Wood *et al.*, 1997; Grunewald *et al.*, 2003). Yet several unique features of the HCMV capsid such as the role of the smallest capsid protein in production of infectious virions suggest that there are differences in the assembly of capsids of these two viruses. Although the atomic structure of HCMV capsid proteins has not been determined, several reports have characterized some of the more relevant protein–protein

Table 20.1. Proteins of the capsid of β-herpesviruses: comparison with HSV

Protein	HSV	HCMV[1]	HHV-6; HHV-7[2]
Major capsid protein	Vp5 (UL119)	MCP (UL86)*	U57
Small capsid protein	Vp26 (UL35)	SCP (UL48A)*	U32
Minor capsid protein[3]	Vp 23(UL18)	MnCP (UL85)*	U56
Minor capsid protein[3]	Vp19c (UL38)	MnCP-bp (UL46)*	U29
Assembly protein	Vp 22a (UL26.5)	Assembly protein (UL80.5)	–
Assembly protein precursor	Vp21(UL26)	Assemblin precursor$_{COOH}$ (UL80a)	U53
Assembly protein precursor	Vp24 (UL26)	Assemblin (UL80a)*	–
Portal protein	Vp (UL6)	UL104	U76

[1] The * indicates capsid proteins that have been demonstrated in infectious virions. Pre-B capsids are thought to contain products of both the UL80a and UL80.5 orfs, mature virions have also been shown to contain products of the UL80a orf, but not UL80.5. Modified from Gibson (Gibson, 1996)

[2] Homologous orf from HHV-6 and HHV-7 based on published nucleotide sequence (Dominguez *et al.*, 1999; Gompels *et al.*, 1995; Nicholas, 1996)

[3] The MnCP (UL85) is also referred to as the triplex dimer protein or TR-2 and the MnCP-bp is referred to as the triplex monomer protein or TR-1.

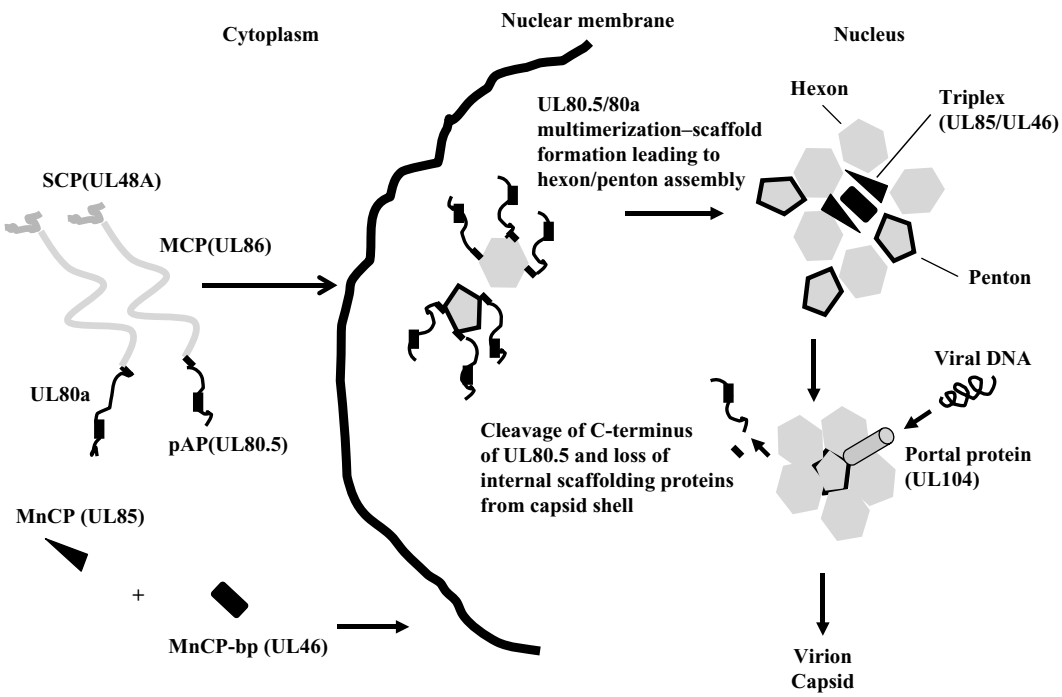

Fig. 20.1. Model of HCMV encapsidation. (1) The capsid proteins MCP, SCP, and products of the UL80a and UL80.5 orf that contain a MCP binding domain are thought to interact in the cytoplasm and are then translocated into the nucleus of the infected cell. The MnCP (UL85) and MnCP-bp (UL46) are also translocated into the nucleus but not as part of the SCP/MCP/UL80a,80.5 complex. (2) Once in the nucleus, self-interaction domains in the products of the UL80a (assemblin precursor) or the UL80.5 (assembly protein) lead to formation of pentons and hexons and the generation of the capsid scaffold. These structures then interact with the triplex formed between MnCP and MnCP-bp and pre-capsid shells form. (3) Unit length viral DNA is inserted into the capsid shell by proteins of the packaging/terminase complex including the proteins encoded by the UL89 and UL56 orfs through the capsid portal formed by the products of the UL104 orf. Coupled with the entry of viral DNA is the extrusion of the assembly protein (and forms of the assembling precursor secondary to cleavage at the carboxyl domain of these proteins). Nomenclature and model adapted from Gibson (Gibson 1996).

interactions that appear to be required for HCMV capsid assembly. The identity of participating cellular proteins and in many cases the temporal order of protein–protein interactions that lead to capsid formation remain undefined. The template for HCMV capsid assembly can be derived from the pioneering studies of HSV capsid assembly from several laboratories (Heymann *et al.*, 2003; Newcomb *et al.*, 1994, 1996, 1999, 2001; Oien *et al.*, 1997; Tatman *et al.*, 1994; Zhou *et al.*, 2000).

The initial steps of capsid assembly involve formation of a scaffold for the generation of the capsid subunit and the pre-capsid structures, protein interactions between the MCP and UL80a and UL80.5, and possible interactions between MCP and SCP. The scaffolding protein(s) of HCMV has been identified as gene products of UL80a and UL80.5 ORFs (Welch *et al.*, 1991a, b). These genes are organized as nested, in-frame 3-coterminal genes that give rise to four transcripts and four gene products with common carboxyl termini (Welch *et al.*, 1991a). The largest transcript is derived from UL80a and encodes the proteinase precursor which includes the HCMV proteinase in the amino terminal half of the precursor and the assembly protein in the carboxyl terminal half (Welch *et al.*, 1991a,b). Self-cleavage of UL80a by the proteinase function leads to generation of the assemblin and a COOH terminal fragment that overlaps with UL80.5 (Welch *et al.*, 1991b). The role of this protein in capsid assembly appears to be analogous to that reported for the HSV scaffolding protein, VP24 (Liu and Stinski, 1992b; Preston *et al.*, 1992, 1994; Tatman *et al.*, 1994). A second protein, the assembly protein, is the product of UL80.5, a nested ORF collinear with the carboxyl terminus of UL80a. The proteins encoded by the longest ORF, UL80a, and the UL80.5 ORF both express a conserved domain (CCD) at the extreme carboxyl terminus that has been shown to interact with the HCMV MCP (Gibson, 1996; Wood *et al.*, 1997). This interaction is required for nuclear translocation of the MCP (and presumably SCP) because the MCP lack a nuclear localization signal and its mass of 150 kDa would exclude it from passive nuclear entry. Consistent with this prediction is the finding that expression of MCP in the absence of other viral proteins results in cytoplasmic localization (Beaudet-Miller *et al.*, 1996; Lai and Britt, W. J., 2003; Nicholson *et al.*, 1994). Because a conserved functional nuclear localization signal is present in the C-terminal domain of UL80.5 as well as some forms of UL80a, interaction between these proteins and MCP results in the nuclear translocation of MCP (Beaudet-Miller *et al.*, 1996) as depicted in Fig. 20.1. An amino-terminal conserved domain (ACD) can be found in the amino terminal domain of UL80.5 and the corresponding cleavage product of UL80a. This domain promotes self-interaction and has been suggested to lead to the generation of UL80a and UL80.5 multimers (Beaudet-Miller *et al.*, 1996; Gibson, 1996; Wood *et al.*, 1997). The self-interaction, together with interactions with MCP, is thought to catalyze the association of MCP into multimers leading to the formation of intranuclear hexons and pentons (Fig. 20.1) (Gibson, 1996). Interestingly, the SCP has also been shown to interact with the MCP in the absence of other viral proteins (Lai and Britt, W. J., 2003). The sequences of SCP that are responsible for this interaction have been mapped but the functional significance of this interaction has not been studied in the context of replicating virus (Lai and Britt, W. J., 2003). The interaction between these two abundant and essential components of the capsid raises several important questions, including if the observed interaction between the SCP and MCP is critical for structural events leading to capsid assembly or for a downstream event in virion assembly such as capsid/tegument interactions required for capsid tegumentation and virion assembly. Interactions between the products of the UL85 and UL46 have also been demonstrated by two-hybrid systems (Gibson *et al.*, 1996). This interaction is required for the formation of the triplexes of the capsid and presumably results in a conformational change that allows their positioning between capsomeres. The interacting sequences of this set of capsid proteins have not been unequivocally mapped, perhaps as a result of the limited solubility of the protein products of UL46 and their aggregation by heating (Gibson *et al.*, 1996).

Capsid maturation and DNA packaging

Once the immature shell of the capsid is formed, viral DNA must be packaged into this immature capsid or precapsid to generate a mature nucleocapsid. In studies of HSV the packaging of unit length viral DNA takes place in a well described pathway in which capsid maturation and DNA packaging appear to be coupled (Heymann *et al.*, 2003; Steven and Spear, 1997). However, capsid assembly and most aspects of capsid maturation can take place in the absence of viral DNA as evidenced by HSV capsid formation in a virus-free and in an in vitro cell free system (Newcomb *et al.*, 1994; Tatman *et al.*, 1994). The final step in HCMV capsid maturation, based on models of HSV capsid assembly, involve proteolytic cleavage of the carboxyl terminal MCP binding domain (M domain) of UL80.5 and M domain containing forms of UL80a in the shells of the immature B-capsids and including any full length UL80a that may be present in these precapsid forms (Gibson, 1996). The loss of the UL80 encoded scaffolding structures appears to be coupled to viral DNA packaging (Gibson, 1996; Lee *et al.*, 1988). Unit length viral DNA enters the maturing

capsid presumably through an asymmetric site of the capsid structure that based on the HSV model, contains a portal protein. In the case of HCMV, the portal protein has been proposed to be encoded by the UL104 product (Komazin *et al.*, 2004). Entering viral DNA is thought to lead to extrusion of the cleavage products of the UL80 from the virion capsid (Gibson, 1996). It is likely that fragments of UL80a or 80.5 remain in the capsid; however, their role in the maintenance of the structure of the virion capsid is unclear.

The packaging of viral DNA is mediated through virus-encoded protein recognition of two conserved sequence motifs, the *pac-1* and *pac-2* sequences, that are located in the *a* sequence at each end of the viral genome (Mocarski and Tan Courcelle C., 2001). The virus proteins mediating packaging comprise the viral terminase complex that consists of at least two proteins, the products of the UL56 and UL89 orfs, that function together with the UL104 portal protein (Bogner *et al.*, 1998; Giesen *et al.*, 2000; Scheffczik *et al.*, 2002; Scholz *et al.*, 2003). The product of UL56 is a 130 kDa protein that has been shown to bind to AT rich sequences in the *pac* sequences and also to have nuclease activity, both activities that suggest its role in packaging as well as cleavage of viral DNA (Bogner *et al.*, 1998; Scholz *et al.*, 2003). More recently, studies from this same laboratory have suggested that the product of the UL89 ORF, a 75 kDa protein, is actually responsible for the DNA cleavage activity that has been assigned to the terminase complex (Scheffczik *et al.*, 2002). Interestingly, the activity of several members of a group of antiviral drugs that have in common a benzimidazole core structure has been shown to map to UL89 and UL56 (Krosky *et al.*, 1998). Although these two proteins are essential for the recognition and packaging of unit length viral DNA, several unexpected findings in studies of the mechanism of action of the benzimidazole antiviral drugs, including maribavir, have suggested that other virus-encoded proteins could be present in this cleavage-packaging complex.

Nuclear tegumentation and nuclear egress

Reports from several laboratories in the early 1990s resolved several inconsistencies in the literature of alphaherpesvirus envelopment. These studies lead to a model in which virion capsids are initially enveloped at the nuclear envelope and following a de-envelopment step at the outer nuclear membrane are delivered to the cytoplasm for final envelopment at cytoplasmic membranes (Brack *et al.*, 1999; Jones and Grose, C., 1988; Skeppner *et al.*, 2001; Wang *et al.*, 2000; Whealy *et al.*, 1991; Zhu *et al.*, 1995). These studies of capsid egress from the nucleus of HSV (or PRV, VZV) infected cells have been illuminating, but differences in cellular tropism, replication, virion protein composition and virion morphology between betaherpesviruses and alphaherpesviruses suggest that virion assembly distal to the nuclear events of encapsidation could be significantly different. Furthermore, more recent studies have demonstrated that a significant number of tegument proteins encoded by HCMV lack sequence homologues in HSV, and in some cases virion tegument proteins of one betaherpesvirus (HCMV) do not have homologues in other betaherpesviruses, including other cytomegaloviruses. Finally, the changes in the size and morphology of the nucleus that are observed in HCMV infected primary fibroblasts are characteristic of the cytopathic effect of this virus and distinct from changes in cells infected with HSV or PRV. As a result, investigators studying the assembly of HCMV and other betaherpesviruses have postulated models for nuclear egress and envelopment that could be specific to this group of viruses.

Perhaps the most poorly understood aspect of the assembly of herpesviruses is the tegumentation of virion particle. In addition to the undefined number of proteins in the tegument, it remains to be determined whether the essential function of individual tegument proteins is regulatory such as pp71 (UL82), interference with host responses such as pp65 (UL83), structural such as pp28 (UL99), or in some cases multifunctional (Baldick *et al.*, 1997; Browne *et al.*, 2003; Kalejta and Shenk, T., 2003; Liu and Stinski, 1992a; Silva *et al.*, 2003). Thus, genetic deletions affecting ORFs encoding tegument proteins that result in loss of infectivity could be ascribed to several possible mechanisms other than a block in the assembly of an infectious particle secondary to the loss of an essential structural protein. Furthermore, the trafficking of nuclear tegument proteins that eventually are incorporated into the infectious particle remains almost completely unstudied. Examples of the different distribution of HCMV tegument proteins are illustrated in Table 20.2. The most striking aspect of the differing cellular localization of this subset of tegument proteins is that virus assembly presumably requires organization of the tegument to insure ordered incorporation of tegument proteins as well as maintenance of essential protein–protein interactions.

This can be readily explained by a radial distribution of tegument proteins in the particle and by the sequential addition of these proteins during nucleocapsid transit from the nucleus through cellular compartments that are used for final assembly. However, cryoelectron microscopic analysis of the virion structure, although consistent with the sequential acquisition of tegument proteins, has not provided definitive evidence for such a pathway leading to tegument assembly (Chen *et al.*, 1999; Trus *et al.*, 1999).

Table 20.2. Cellular localization of a subset of HCMV tegument proteins

		Cellular localization in productive infection		
ORF	Protein	Nucleus	Cytoplasm	Nucleus and cytoplasm
UL25	ppUL25	−	+	−
UL26	ppUL26	+	−	−
UL32	pp150	+[2]	+	−
UL48	pp200	+	−	−
UL50	p35	+	−	−
UL53	pp38	+	−	+[1]
UL69	ppUL69	+	−	−
UL82	pp71	+	−	?
UL83	pp65	+	−	+[1]
UL94	pp36	+	−	−
UL99	pp28	−	+	−

[1] This set of tegument proteins remains localized exclusively to the nucleus until late in the replication cycle and then can be found in both the nucleus and cytoplasm.
[2]Nuclear localization of pp150 can be demonstrated with different conditions of fixation.

Thus, the assembly of the tegument layer of HCMV remains unclear and may involve nuclear tegumentation and de-tegumentation as has been proposed for PRV (Fuchs *et al.*, 2002; Granzow *et al.*, 1997, 2001). Studies of the alphaherpesvirus PRV have demonstrated that nuclear viral capsids are essentially free of detectable tegument proteins and acquire tegument entirely in the cytoplasm (Granzow *et al.*, 1997, 2001). From a combination of elegant electron microscopic analyses and studies of viral deletion mutants, Mittenleiter and colleagues have proposed compelling arguments that the PRV capsid interacts with non-structural virus-encoded nuclear proteins that facilitate capsid envelopment and de-envelopment at the nuclear envelope (Fig. 20.2) (Mettenleiter, 2002). The virus-encoded proteins UL31 and UL34 of PRV are thought to be essential for nuclear egress and have been shown to be localized to the nucleus of infected cells but cannot be detected in the virion (Fuchs *et al.*, 2002). The viral and/or cellular proteins that participate in these nuclear fusion events, other than UL31 and UL 34, have not been identified. Although this model of PRV nuclear egress is consistent with a number of observations from these and other investigators, other examples of tegument protein trafficking fail to fit precisely with this model. An example is the HSV tegument

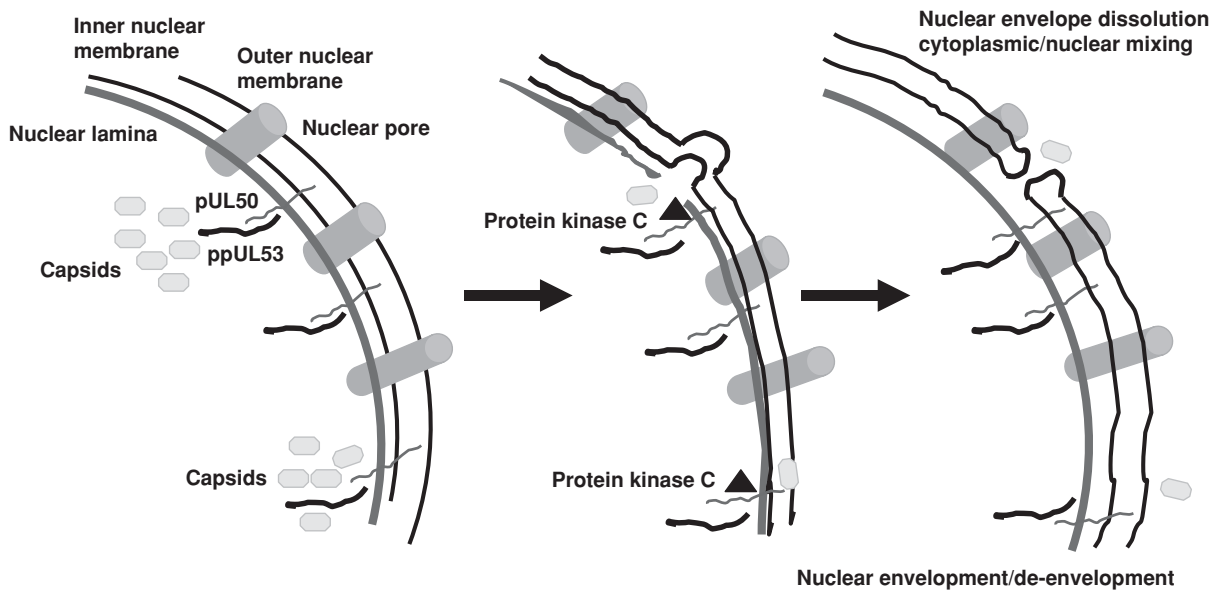

Fig. 20.2. Two models of nuclear egress of the HCMV capsid. The structure of the bi-leaflet nuclear envelope and underlying nuclear lamina is shown. Nuclear pore complexes are depicted as cylinders. Focal accumulation of intranuclear capsids adjacent to inner nuclear membrane domains containing viral proteins encoded by UL50 and UL53, with UL50 shown inserting into inner nuclear membrane. In the top model, nuclear lamina dissolution secondary to lamin phosphorylation associated with localization of protein kinase C activity leads to nuclear envelope herniation, nuclear cytoplasmic mixing and eventual capsid release into the cytoplasm (Muranyi *et al.*, 2002). The nuclear envelope rapidly reseals resulting in only transient nuclear/cytoplasmic mixing. In the bottom model, the focal accumulation of capsids and their interaction with as yet unidentified cellular or virus-encoded proteins leads to a fusion with the inner nuclear envelope protein, translocation into intranuclear space and a second event leading to budding into the cytoplasm. Note the second mechanism of capsid egress is also postulated to require nuclear lamina dissolution.

protein Vp22 (UL49) that has been shown localized to the nucleus of the infected cell and cannot be detected on immature perinuclear capsids, yet it is eventually incorporated into the virion as a tegument protein, presumably during cytoplasmic tegumentation (del Rio *et al.*, 2002). Early electron microscopic findings indicated that the cytoplasmic forms of HCMV contained a considerably thicker tegument as compared to HSV (and presumably PRV) and it is well known that numerous HCMV nuclear tegument proteins are present in the virion. Thus, it is likely that nuclear egress of the HCMV capsid is more complex than the models proposed for alphaherpesviruses. This HCMV pathway of nuclear egress could involve nuclear tegumentation, de-tegumentation, and a final tegumentation that would incorporate virion tegument proteins that localize to the nucleus, or even more simply, both nuclear and cytoplasmic tegumentation.

Early studies of HCMV egress noted that virion structural proteins could be localized to the nuclear matrix of virus infected cells (Sanchez *et al.*, 1998). The nuclear matrix of eurkaryotic cells can be viewed as the cytoskeleton of the nucleus. This nuclear structure has been shown to be a site of transcription and DNA replication, including for DNA viruses (Pombo *et al.*, 1994; Schirmbeck *et al.*, 1989). Major components of the nuclear matrix include the nuclear lamins, a group of cellular proteins that represent the intermediate filaments of the nucleus (Stuurman *et al.*, 1998). Nuclear lamins are known to play an important role in the integrity of the nuclear envelope and have been shown to link the nuclear matrix with the inner membrane of the nuclear envelope through their interactions with a number of integral membrane proteins in the inner nuclear membrane, thus creating what is referred to as the nuclear lamina. The finding of a specific interaction of HCMV virion tegument proteins with the nuclear matrix, and in particular lamin B, raised the possibility that this interaction could represent a pathway of nuclear egress of tegumented capsids (Sanchez *et al.*, 1998). Studies have demonstrated that nuclear lamin phosphorylation and dephosphorylation can be coupled with loss of lamin structure and, in some cases, the disruption of the nuclear envelope (Steen *et al.*, 2000; Stuurman *et al.*, 1998). Lamin phosphorylation has been demonstrated in HCMV infected cells and it was initially suggested that either an intrinsic kinase activity of an HCMV encoded protein or a cellular kinase activity localized to regions adjacent to the nuclear envelope could account for phosphorylation of nuclear lamins (Muranyi *et al.*, 2002; Radsak *et al.*, 1991). The focal loss of nuclear envelope integrity has been suggested to contribute to the nuclear egress of the HCMV capsid (Gallina *et al.*, 1999; Sanchez *et al.*, 1998). However, it should be noted that loss of lamin structure alone cannot account for loss of nuclear membrane integrity.

Consistent with proposed mechanisms of egress of HCMV that include a focal loss of nuclear membrane integrity have been studies of the Vpr protein of HIV-1. Expression of this virus-encoded protein has been shown to be associated with the focal loss of nuclear membrane integrity and mixing of cytoplasmic and nuclear proteins (de Noronha *et al.*, 2001). A recent study utilizing murine CMV as a model system reported similar findings by demonstrating that two non-structural nuclear proteins encoded by m50 and m53 could localize host protein kinase C activity to an area adjacent to the nuclear envelope (Muranyi *et al.*, 2002). The redistribution of protein kinase C activity resulted in the phosphorylation of lamins A and C at levels that could be associated with loss of structural integrity of the inner nuclear membrane (Muranyi *et al.*, 2002). The m50 and m53 are sequence homologues of the alphaherpesvirus UL34 and UL31, respectively, and are believed to represent the functional homologues of these proteins. Similar to the alphaherpesvirus UL34 protein, the m50 product is a type II membrane protein that contains motifs with limited homology with known integral membrane proteins of the nuclear envelope (Muranyi *et al.*, 2002). Because both m50 and m53 encode essential functions for virus replication, this finding suggests that nuclear egress of CMVs could require focal disruption of the nuclear lamins leading to the loss of the integrity of the inner nuclear membrane. The homologous proteins of HCMV, pUL50 and ppUL53, have also been shown to be essential for virus replication and interestingly, ppUL53 can be detected in the virion tegument, suggesting that it has an additional role in virus assembly other than its proposed function in facilitating nuclear egress (Dal Monte *et al.*, 2002). Studies of ppUL53 trafficking indicate that, in the absence of other virus-encoded proteins, it is expressed only in the nucleus but that it can be detected in both the nucleus and the virus assembly compartment in virus infected cells late in infection (Dal Monte *et al.*, 2002) (W. Britt, unpublished data). Thus, in contrast to findings in the PRV, one of the essential nuclear virus-encoded proteins that is thought to be required for modification of the nuclear envelope is also found within the tegument of the mature virion. It is unclear if HCMV pUL50 is present in the virion tegument. Expression of the abundant HCMV virion tegument protein pp65 (UL83) is also restricted to the nucleus of infected cells secondary to a functional bipartite nuclear localization signal until late in infection when it is distributed in the cytoplasm and can be detected predominantly in the assembly compartment (Sanchez *et al.*, 2000a). These findings would indicate that either HCMV

virions are partially tegumented in the nucleus or that nuclear tegument proteins have trafficking pathways to sites of tegument assembly in the cytoplasm.

Other models of nuclear egress have been proposed yet insufficient data is available to determine their validity. In studies of HHV-7, Frenkel and co-workers described an intranuclear, cytoplasmic vacuole or invagination that appeared to be a site of nuclear budding of the mature capsid (Roffman *et al.*, 1990). This structure designated a tegusome and was identified by electron microscopic analysis of infected cells (Roffman *et al.*, 1990). This structure provided an explanation consistent with the incorporation of virus-encoded nuclear proteins into the tegument of the mature particle. Similar structures have not been identified in other betaherpesviruses.

A pathway of egress of herpesvirus capsids from nucleus that unifies available data has yet to be described. However, it is clear that these viruses have devoted at least some of their genomic information to encode proteins that can either directly modify key structures of the nuclear envelope or recruit cellular functions to the nuclear membrane. These findings would suggest that immature capsids (and possibly tegumented capsids) could exit the nucleus through focal herniations of nuclear membrane as proposed for the function of Vpr of HIV. Alternatively, translocation of the capsid across the nuclear envelope into the cytoplasm of the infected cell could require a membrane fusion event, a mechanism that is favored by the bulk of the literature describing alphaherpesvirus assembly.

Cytoplasmic tegumentation and envelopment

Final tegumentation and envelopment of the infectious betaherpesvirus particle appear to occur exclusively in the cytoplasm of the infected cell. Studies that have described the trafficking of virion tegument and envelope proteins have led to several proposed models of cytoplasmic assembly of the infectious virion. Two studies of HCMV utilizing cryoelectron microscopy indicated that the tegument layer adjacent to the capsid exhibited aspects of iscosahedral symmetry and, from estimates of the mass of the presumed tegument protein that occupied this layer, one group of investigators argued that the protein adjacent to the capsid was pp150 (UL32) (Trus *et al.*, 1999); however, the identity of the protein was not pursued either biochemically or immunologically. The suggestion that the tegument protein pp150 was adjacent to the capsid raised the possibility that this virion protein was acquired during nuclear egress of the capsid. The distribution of pp150 within infected cells was initially proposed to be nuclear and cytoplasmic, although a subsequent study that employed a larger number of viral and cell markers suggested that pp150 was expressed only in the cytoplasm of infected cells (Hensel *et al.*, 1995; Sanchez *et al.*, 2000a). In this latter study, pp150 was used to define an isolable cellular compartment that was designated as the assembly compartment (Sanchez *et al.*, 2000a). This compartment was shown to be localized to a juxtanuclear site that was in close proximity to the microtubular organizing center (MTOC) of infected cells (Sanchez *et al.*, 2000a). Subsequent studies from other laboratories have also identified this cytoplasmic structure (Dal Monte *et al.*, 2002; Silva *et al.*, 2003). A number of both nuclear and cytoplasmic localized tegument proteins have been shown to localize to the assembly compartment, including many of the proteins listed in Tables 20.2 and 20.3. Isolation of this compartment by centrifugation of cell lysates over density gradients allowed the identification of both tegument and envelope proteins, including processed glycoprotein B (gB, UL55) within in this compartment, consistent with this compartment being a site of virus envelopment and assembly. The trafficking and accumulation of virion proteins and subviral structures to this site is not understood; however, recent studies of tegument protein trafficking coupled with a greater understanding of glycoprotein trafficking are beginning to suggest model pathways for its formation within infected cells and may represent a cytoplasmic inclusion identified in early studies of HCMV morphogenesis.

Tegument protein trafficking and incorporation into the particle

Although it is likely that understanding the trafficking of virion tegument proteins will be key to understanding the assembly of the infectious particle, little is known about the localization of these proteins to the assembly compartment. One structural tegument protein for which some features of its intracellular trafficking are known is pp28 (UL99). Studies demonstrated that this small (191 amino acid) myristylated protein is membrane associated and when expressed in the absence of other viral proteins, is retained in the ER/Golgi intermediate compartment (ERGIC) (Sanchez *et al.*, 2000b). In virus infected cells, pp28 is transported to the assembly compartment, where it localizes with envelope glycoproteins and with other tegument proteins including pp150 (Sanchez *et al.*, 2000a; Silva *et al.*, 2003). Recombinant viruses in which the gene encoding UL99 has been deleted, the reading frame has been interrupted, or the protein mistargeted in the infected cell by deletion of the myristylation site are non-infectious and non-enveloped particles were observed in the cytoplasm of cells infected with the mutant pp28 deletion virus (Britt

Table 20.3. HCMV envelope glycoproteins

Glycoprotein	Orf[1]	Complex formation	Essential for infectivity	Proposed function
gB	UL55(U39)	oligomer	Yes	Attachment/fusion
gH	UL75 (U48)	gH/gL/gO	Yes	Fusion/penetration
gL	UL115(U82)	gH/gL/gO	Yes	Fusion/penetration
gO	UL74(U100[2])	gH/gL/gO	Yes	Fusion/penetration
gM	UL100(U72)	gM/gN	Yes	Unknown
gN	UL73(U46)	gM/gN	Yes	Unknown
gpTRL10	TRL10	Unknown	No	Unknown
gpUL132	UL132	Unknown	No	Unknown

[1] Homologous orf from HHV-6 and HHV-7 based on published nucleotide sequence are shown in parentheses (Dominguez *et al.*, 1999; Gompels *et al.*, 1995; Nicholas, 1996).
[2] Note the HHV-6 functional homologue of HCMV gO has been identified as the glycoprotein encoded by HHV-6 orf U100 and has been designated gQ (Mori *et al.*, 2003).

et al., 2004; Jones and Lee, 2004; Silva *et al.*, 2003). Interestingly, the virion assembly compartment was formed in cells infected with the UL99 deletion mutant, indicating that this protein was not required for trafficking of other tegument and presumably envelope proteins to this cytoplasmic compartment. The trafficking of other tegument proteins, including nuclear tegument proteins, to the assembly compartment has not been studied in sufficient detail to allow investigators to develop a model of HCMV tegument assembly.

Envelope glycoprotein trafficking and envelopment of the particle

Similar to the uncertainties that surround the structure and composition of the tegument, the envelope of HCMV remains poorly defined. Although the analysis of the coding sequence of HCMVs suggest that as many as 50 ORFs could encode proteins with N-linked carbohydrate modification, to date, some eight experimentally defined virus specific glycoproteins have been shown to be present in the virion envelope (Table 20.3). At least six of these have been shown to exist as disulfide-linked oligomers (gB, gH/gL/gO; gM/gN) in the virion, a characteristic of CMVs that at first glance appears unique in the herpesvirus family. Formation of these disulfide-linked oligomers takes place in the ER prior to transport of these complexes through the secretory pathway. When expressed in the absence of other viral proteins, glycoproteins (gB) or complexes of glycoproteins (gH/gL/gO; gM/gN) listed in Table 20.3, with the exception of gpTRL10, traffic to Golgi and post-Golgi compartments.

Many of these glycoproteins have been shown to contain well-described signals within their cytoplasmic domains that enable them to utilize the cellular secretory pathway for intracellular trafficking. Examples of these signals include phosphorylated amino acid residues and acidic amino acid clusters that are recognized by cellular adaptor proteins including PACS-1 that localize proteins to the TGN, tyrosine and di-leucine signals that promote glycoprotein retrieval from the cell surface and possibly from other cellular membranes through their interactions with cellular adaptor proteins, and likely other motifs that facilitate interactions with tegument proteins (Crump *et al.*, 2003; Jarvis *et al.*, 2002, Tugizov *et al.*, 1999). Although the role of these various signals on individual glycoproteins has been shown in many cases to function in intracellular trafficking as predicted based on studies of similar signals on other viral glycoproteins, it remains unclear what role these signals play in the assembly and infectivity of the mature virion. As an example, it has been argued that gB in the envelope of infectious virus was derived from cell surface gB retrieved from the cell surface by endocytosis and targeted to the TGN, a presumed site of virus assembly (Radsak *et al.*, 1996; Tugizov *et al.*, 1999). Yet recent studies have shown that mutation of this targeting signal in gB has no effect on the phenotype of the mutant virus when compared to wild-type virus (Jarvis *et al.*, 2002). This latter finding raises several possible interpretations such as a redundancy of targeting signals in the cytoplasmic domain of HCMV virion glycoproteins or that additional intracellular pathways are operative in cells infected with this virus that enable the virion glycoproteins to localize in the assembly compartment. However, it is important to note that several of these glycoproteins have very conventional targeting motifs that are conserved in their cytoplasmic domains, a finding that suggests that well-described cellular pathways of viral

glycoprotein localization are utilized during virion assembly. A complete discussion of the intracellular trafficking of these proteins is beyond the scope of this section but, because of the potential cooperativity between proteins and redundancy of function, many of the observations made in the study of individual HCMV glycoproteins in isolation from virus infection could be of limited value in definition of their role in assembly and function in the mature virion. The development of recombinant systems that enable investigators to insert specific mutations should help address these aspects of HCMV envelopment.

The mechanisms leading to final envelopment of the infectious HCMV or other betaherpesvirus particles have not been described. In fact, localizing the virion tegument proteins and envelope glycoproteins to an isolable cellular compartment suggests that envelopment takes place after tegumentation of the nucleocapsid. The recent findings that described unenveloped particles in the cytoplasm of cells infected with the UL99 (pp28) deletion virus supports a working model of envelopment that includes budding of a tegumented capsid through a membrane that contains viral envelope glycoproteins (Silva *et al.*, 2003). The source of the membrane structure and the mechanisms that localize the large number of glycoproteins to this single membrane have not been well described but recent studies of the trafficking of HCMV UL33 and UL27 have suggested that virions were wrapped in membrane tubules as well as budding into multivesicular bodies, structures connected with the endosomal system (Fraile-Ramos *et al.*, 2001). The location of these membranous structures in a juxtanuclear compartment and the differentiation of this compartment from Golgi and TGN are consistent with previous studies of the virus assembly compartment identified in virus infected cells (Fraile-Ramos *et al.*, 2001; Sanchez *et al.*, 2000a; Homman-Londiyi *et al.*, 2003). Previous studies also suggested that HCMV was enveloped in an endosomal compartment (Tooze *et al.*, 1993). Other enveloped viruses, most notably retroviruses, have been shown to utilize a pathway that includes budding into late endosomal compartments during their assembly (Amara *et al.*, 2003; Pelchen-Matthews *et al.*, 2003). If such cellular structures are ultimately shown to be a site of HCMV envelopment (budding), it could follow that HCMV exits the infected cell by a similar exocytic pathway (Gould *et al.*, 2003).

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21

Viral modulation of the host response to infection

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Betaherpesviruses such as HCMV dramatically affect host cell physiology and encode a wide variety of functions that modulate the infected host cell as well as the immune response (Mocarski, 2002, 2004). Major structural and nonstructural proteins modulate host cell transcriptional repression (Saffert and Kalejta, 2006, Tavalai *et al.*, 2006), cell-intrinsic responses (Abate *et al.*, 2004; Goldmacher, 2004), responses to interferon (Child *et al.*, 2004; Khan *et al.*, 2004) and natural killer (NK) lymphocytes (Lodoen and Lanier, 2005), and adaptive antibody or T-lymphocyte immunity (Chapter 62). The host immune components that are targets of modulation by HCMV are the same host functions that are important in suppressing virus infection, suggesting that the balance between host clearance and viral escape mechanisms dictates many aspects of viral pathogenesis. By reducing the overall impact of antiviral defenses, HCMV seems to be able to escape the full brunt of host innate and adaptive immunity, thereby allowing the virus to persist. It now appears that an overwhelming majority of viral gene products are dedicated to modulation of host cell and immune modulation (Chapter 15). The overwhelming majority (~100 gene products) may be implicated in modulation because they are dispensable for replication in cultured fibroblasts (Dunn *et al.*, 2003; Yu *et al.*, 2003).

During infection, HCMV and other cytomegaloviruses have a striking impact on cellular gene expression, cell cycle progression, and cellular behavior. More limited information suggests that roseolaviruses, HHV-6A, HHV-6B, and HHV-7 have a similar impact on cells (Chapters 18 and 47). In HCMV several major phases of cellular modulation occur. An interferon-like stimulation of host cells immediately follows virus entry due to the effect of virus particles (see Chapter 16) even though cellular alarm systems involved in IRF-3 and apoptosis are defused. Activation of viral gene transcription by derepression of histone deacetylase activity underlies expression of viral gene products (see Chapters 17 and 18). A dysregulation of cell cycle progression follows early viral gene expression (see Chapter 18) and extends through a pseudo-S phase, and can culminate in pseudomitosis (a property that varies among viral) (Hertel and Mocarski, 2004). A poorly understood process results in the death of the host cell following several days of productive replication. All of these likely vary with host cell type and susceptibility, and all of these may impact replication efficiency. Modulation of host cell and host cell behavior is also a factor in pathogenesis, possibly mediating important stages and cell type specific events during acute and chronic disease in the infected host. Thus, specific HCMV-encoded proteins have been shown to modulate interferon regulated factor (IRF)-3, host cell susceptibility to apoptosis, induction and activity of host cytokines and interferons, stability and cell surface expression of both classical and non-classical major histocompatibility complex (MHC) proteins, and many key processes involved in host cell signaling, gene expression, and metabolism (Mocarski, 2002, 2004; Mocarski and Courcelle, 2001). Together, these broad modulatory capabilities likely contribute to the success of the betaherpesviruses as ubiquitous pathogens. Although many interface with known cellular growth control points in ways that are better studied in the gammaherpesviruses, little evidence supports an oncogenic role for this group of viruses. The impact of HCMV on cells has reinforced the notion that the virus may be involved in acute and chronic diseases affecting the vasculature, and is supported by experiments with rodent cytomegaloviruses. This chapter will focus on viral modulation of the cellular response to betaherpesvirus infection, focusing primarily on HCMV due to the information available.

Modulation of histone deacetylase activity

HCMV replication is stimulated when permissive cells are treated with inhibitors of histone deacetylases (Murphy *et al.*, 2002) or when virus is propagated in cells in which expression of histone deacetylases has been inhibited (Tavalai *et al.*, 2006). Two viral gene products have been implicated in derepression. One major tegument protein, ppUL82 (pp71), also known as the virion transactivator, appears to inactivate Daxx (Cantrell *et al.*, 2005; Everett, 2006; Hofmann *et al.*, 2002; Ishov *et al.*, 2002; Staffert and Kalejta, 2006). The major immediate early protein, IE1-p72, long known to disrupt nuclear domain 10 (ND-10) sites early after infection (Korioth *et al.*, 1996), has been ascribed a role in inactivate a family of promyelocytic leukemia protein (PML) proteins, that also act as repressors of viral gene expression (tavalai *et al.*, 2006).

Modulation of IRF–3

HCMV entry into cells is associated with a dramatic induction of NF-κB (Yurochko *et al.*, 1997a) that is likely to drive the interferon-like response of cells exposed to infectious virus, viral particles, or soluble envelope glycoprotein gB (Simmen *et al.*, 2001; Yurochko *et al.*, 1997a; Zhu *et al.*, 1997, 1998). Although the interferon β transcript is induced in virus-infected cells, even following high MOI infection, interferon itself is only produced in cell cultures subjected to low MOI infection (Rodriguez *et al.*, 1987; Zhu *et al.*, 1997). Several investigators, have described the induction of IRF-3 immediately after infection of permissive fibroblasts (Boehme *et al.*, 2004; Browne and Shenk, 2003; Navarro *et al.*, 1998; Preston *et al.*, 2001), using a variety of viral strains and infection conditions. Induction has been associated with the appearance of a novel IRF-3 complex (Navarro *et al.*, 1998). In contrast, experiments that included both uniform high MOI and use of a monoclonal antibody to detect IRF-3 demonstrated that wild type strains of HCMV fail to induce IRF-3 (Abate *et al.*, 2004), a property that may be shared by rhesus CMV (DeFilippis and Fruh, 2005). Experimental differences in MOI and time post infection, as well as in choice of viral strains and strain variants, may contribute to differences in IRF-3 activation and level of inhibition by pp65 (ppUL83) during infection. Virion pp65 reduces the level of activation of IRF-3 immediately following infection and pp65 expression independent of viral infection is sufficient to inhibit IRF-3 activation by a variety of signals (Abate *et al.*, 2004). In the presence of pp65, there appears to be a transient activation of IRF-3 (Yang *et al.*, 2005) that does not exhibit kinetics consistent with the dramatic impact of virus infection on cells. NF-kB, which has been a topic of study for many years (see below), may also be a target of pp65 depending on experimental conditions (Browne *et al.*, 2003). Even though IRF-3 activation that occurs within 2 h postinfection is likely to be dependent on preformed cytoplasmic protein, the ability of IRF-3 siRNA to increase expression of certain IRF-3 responsive genes and decrease HCMV replication levels has been reported (DeFilippis *et al.*, 2006). The mechanism of IRF-3 regulation over the first few hours of infection will emerge from further mechanistic studies.

Activation of NK-κB and interferon response genes

A classical NF-κB response occurs in two distinct phases following HCMV infection of fibroblasts (Johnson *et al.*, 2001a; Kowalik *et al.*, 1993; Sambucetti *et al.*, 1989; Yurochko *et al.*, 1995). The first phase follows as early as 5 minutes after exposure of cells to virus or virus particles and apparently results from the release of preformed NF-κB mediated by the binding and postattachment events (Boyle *et al.*, 1999; Yurochko *et al.*, 1997a; Netterwald *et al.*, 2004). This initial NF-κB activation may underlie the virion-induced interferon-like response (Browne *et al.*, 2001; Zhu *et al.*, 1997, 1998) and enhance expression of immediate early (IE) genes through the enhancer (DeMeritt *et al.*, 2004). Based on studies with MCMV, the NF-κB sites in the enhancer region may not be essential in all settings (Benedict *et al.*, 2004). NF-κB activation requires phosphorylation-dependent degradation of an inhibitor of NF-κB (IκB), and this degradation is activated in response to infection and continues throughout the infection cycle (Kowalik *et al.*, 1993). Phosphorylation is dependent on a three subunit IκB kinase (IKK) that is both activated and, based solely on the use of chemical inhibitors, required for initiation of viral replication (Caposio *et al.*, 2004) in quiescent fibroblasts. Activation is not critical in actively growing astrocytoma cells (Eickhoff and Cotten, 2005). Deletions made through the enhancer reduce viral replication efficiency in a pattern that suggests a possible role of NF-κB as well as other transcription factor binding sites (Isomura *et al.*, 2004). Another phase of NF-κB activation due to the initiation of NF-κB transcription, allowing continued expression throughout infection (Kowalik *et al.*, 1993; Yurochko *et al.*, 1997b). A physiological role for activation is masked by the relative complexity of events as well as the activities of viral regulatory gene products (Castillo *et al.*, 2000).

The interferon-like and likely NF-kB-dependent activation of cellular gene expression begins within a few hours after virus particle contact with cells (Browne *et al.*, 2001; Simmen *et al.*, 2001) and the response includes a wide range of signaling pathways in addition to those that activate NF-κB (Albrecht *et al.*, 1992; Boldogh *et al.*, 1990, 1991, 1997; Evers *et al.*, 2004; Wang *et al.*, 2003). The production of interferon β itself during HCMV infection is inversely associated with MOI (Rodriguez *et al.*, 1987). Levels of this interferon sufficient to influence the behavior of cells is only induced following exposure of cells to low MOIs or to inactive virus particles (Boehme *et al.*, 2004; Compton, 2004). There is little detectable interferon in the medium or associated with virus particles following high MOI infection (Abate *et al.*, 2004; Rodriguez *et al.*, 1987; Zhu *et al.*, 1997). Interferon has long been known to be relatively ineffective against this group of viruses, whether tested in culture (Holmes *et al.*, 1978) or in patients (Cheeseman *et al.*, 1977). HCMV infected cells do not support interferon receptor signaling or the translation of interferon β transcripts but viral functions that carry out these activities remain to be identified. HCMV deflects major interferon regulated pathways through the action of the closely related viral TRS1 and IRS1 gene products (Child *et al.*, 2002, 2004; Cassady, 2005). These can replace either the vaccinia dsRNA binding protein E3L or the herpes simplex virus-1 (HSV-1) γ34.5 gene product and suppress both protein kinase R (PKR) and the 2–5 oligoadenylate (2–5OAS) synthetase/RNase L system. Despite the lack of obvious motifs, pTRS1 binds dsRNA and includes an unconventional dsRNA-binding domain conserved in pIRS1; however, this domain is not sufficient to rescue E3L mutant virus (Hakki and Geballe, 2005). Thus, TRS1 primarily, but IRS1 secondarily, impede PKR-mediated inactivation of eukaryotic intiation factor 2 as well as the activation of RNase L by products of 2–5A synthetases and the resultant degradation of mRNA and rRNA. IRS1 mutants are fully growth proficient, but growth of TRS1 mutants is reduced (Blankenship and Shenk, 2002; Dunn *et al.*, 2003) and impacts virion assembly (Adamo, *et al.*, 2004).

Impact on the host cell cycle

Betaherpesvirus genomes are transcribed and replicated within the nucleus, the same cellular compartment that regulates the cell cycle and controls apoptosis following DNA damage or aberrant protooncogene expression. Cellular DNA is damaged, the cell cycle is dysregulated, and protooncogene expression is increased following cytomegalovirus infection. DNA damage induced by HCMV includes both randomly distributed and specific chromosomal breaks and gaps (AbuBakar *et al.*, 1988; Deng *et al.*, 1992; Fortunato *et al.*, 2000a). It is not evident that any of these processes requires viral replication.

Cell cycle dysregulation occurs during HCMV productive infection and gives the impression of progression into S phase and mitosis, although cellular DNA synthesis and cell division are blocked. Initial reports of a G2/M arrest, defined by presence of what appeared to be a 4N chromosomal peak (Jault *et al.*, 1995; Lu and Shenk, 1996) were clarified by further work showing host cell DNA content does not increase in infected cells (Bresnahan *et al.*, 1996; Dittmer and Mocarski, 1997; Salvant *et al.*, 1998). Specific inhibition of viral and cellular DNA replication showed that this increase was due to the accumulation of viral DNA (Dittmer and Mocarski, 1997]. Interestingly, viral replication is delayed in cells infected during S phase until after the cell cycle has progressed at least to G2/M, and the majority of cells must apparently cycle to G1 prior to the initiation of viral gene expression (Salvant *et al.*, 1998; Fortunato *et al.*, 2002). It remains possible that the CMV-induced block to cellular DNA synthesis and dysregulation of the cell cycle will vary with each differentiated cell type tested, as detailed studies have not been undertaken in relevant cell types such as endothelial cells, myeloid cells, or epithelial cells. It is notable that studies in MCMV have suggested that induction of apoptosis as a result of viral infection is cell type dependent and that viral inhibitors of apoptosis show cell type specificity (Brune *et al.*, 2001, 2003; Menard *et al.*, 2003).

The structural proteins that modulate host cells most dramatically include two relatively abundant viral tegument proteins, ppUL82 (pp71) (Bresnahan and Shenk, 2000; Kalejta and Shenk, 2002) and pUL69 (Hayashi *et al.*, 2000; Lu and Shenk, 1999). Both are introduced into cells during viral entry. In addition, the IE2 p86 regulatory protein encoded immediately following entry (Murphy *et al.*, 2000; Song and Stinski, 2002) has a dramatic impact on the cell. All three have been characterized for their impact on cell cycle independent of viral replication. pUL69 and IE2 p86 inhibit cell progression past G1 and S, respectively, and pUL82 (pp71) induces quiescent cells to enter the cell cycle. The impact of any of these during viral infection remains poorly understood, but ppUL82 (pp71) may act through derepression of HDACs (Cantrell *et al.*, 2005; Hofmann *et al.*, 2002; Ishov *et al.*, 2002; Saffert and Kalejta, 2006). The situation is complicated by the fact that virus-infected cells do not remain at any distinct stage of the cell cycle but can have characteristics that extend from resting, Go-like to a pseudomitotic state (Hertel and Mocarski, 2004). HHV-7 infection apparently promotes accumulation of polyploid cells with enlarged single cells that have a

polylobated nucleus and >4N genome content (Secchiero *et al.*, 1998).

HCMV and HHV-7 grossly alter cyclin and other cell cycle regulatory protein expression patterns (Fortunato *et al.*, 2000b; Hertel and Mocarski, 2004), as described in Chapter 18. Cell cycle dysregulation is accompanied by increases in cyclin E and cdk2 (Bresnahan *et al.*, 1996; Jault *et al.*, 1995) as well as cdk2 translocation to the nucleus (Bresnahan *et al.*, 1997). Additionally, infected cells accumulate hyperphosphorylated pRB (Jault *et al.*, 1995), consistent with induced E2F-specific gene transcription (Song and Stinski, 2002). Proteins that are normally involved in cellular DNA replication, including PCNA (Dittmer and Mocarski, 1997; Mate *et al.*, 1998) and RPA (Fortunato and Spector, 1998), increase and accumulate within sites of viral DNA replication in the nucleus. Increases in cyclin B and cdc2 activity were first demonstrated in infected cells that appeared to have progressed to the G2/M boundary based on DNA content (Jault *et al.*, 1995), but cyclin B levels clearly increase in fibroblasts arrested by CMV at a G1/S-like boundary (Dittmer and Mocarski, 1997). Despite the presence of regulatory factors required for transition through S phase, cellular DNA synthesis is restricted through virus-specific inhibition of licensing factors (Biswas *et al.*, 2003; Wiebusch *et al.*, 2003). All of these studies provide clear evidence of severe dysregulation by HCMV. Although less well characterized, HHV-7 will likely differ from CMV since cdc2 activity decreases after HHV-7 infection. Similar to CMV, cyclin B increases in HHV-7 infected cells despite a G1/S-like arrest. In conjunction with cell cycle alterations, HCMV infection increases expression of the protooncogenes c-jun, c-fos, c-myc (Boldogh *et al.*, 1990), and the tumor suppressor p53 (Jault *et al.*, 1995; Speir *et al.*, 1994). p53 is a transcription factor that promotes growth arrest or apoptosis in response to cell stress (Haupt *et al.*, 2003) or other regulators of intrinsic cell death such as oncogene activation, nucleotide depletion, hypoxia, redox modulation, and loss of normal cell contacts (Giaccia and Kastan, 1998). p53-induced cell death is primarily due to *trans*-activation of specific apoptosis regulators including several proapototic Bcl-2 family members that promote mitochondria membrane permeability transition. The outcome of p53 activity, growth arrest or induction of apoptosis, depends on many factors including p53 expression levels, p53 co-activators, cell type, and type of stress (Haupt *et al.*, 2003). p53 levels are controlled via Mdm2 (mice) or Hdm2 (humans), which direct degradation via the proteasome. Early during HCMV infection, p53 levels increase due to a decrease in protein turnover (Jault *et al.*, 1995; Muganda *et al.*, 1998; Speir *et al.*, 1994). Further, specific coactivators of p53, such as myc, are induced but apoptosis is not induced, possibly due to accumulation in discrete subnuclear regions colocalizing with the viral DNA polymerase processivity factor, ppUL44 (Fortunato and Spector, 1998), a marker of viral DNA replication compartments (Penfold and Mocarski, 1997). A direct physical interaction with IE2 p86 may inhibit p53-mediated transactivation (Speir *et al.*, 1994). One reported interaction of IE2 p86 and p53 (Bonin and McDougall, 1997), however, was carried out in cells that expressed a non-functional IE2 p86 (Murphy *et al.*, 2000), such that this area needs additional investigation. IE2 p86-deficient viruses fail to replicate at all (Heider *et al.*, 2002; Marchini *et al.*, 2001), and a variety of deletions have been made within the protein coding sequences to delineate regions that are necessary for viral replication (Sanchez *et al.*, 2002; White *et al.*, 2004), as described in Chapter 18. In addition to sequestration of the protein, HCMV may have additional means of negating p53 activity, including the increased expression of inhibitory proteins in the p53-family. p53-mediated transcription of apoptotic genes is influenced by the presence of p73 (Flores *et al.*, 2002). In p53 deficient astrocytoma cells, the presence of p73 confers sensitivity to DNA damaging agents such as cisplatin, and HCMV infection alters sensitivity to such agents (Allart *et al.*, 2002) and increases the expression of a cellular dominant negative isoform of p73 that may interfere with both p53- and p73-dependent activities.

Suppression of apoptosis

Apoptosis is an evolutionarily conserved cellular process that removes cells during infection, development, or homeostasis. Apoptosis is initiated by intrinsic stress or DNA damage within the cell that accompanies infection by obligate intracellular parasites and viruses (Ferri and Kroemer, 2001; Polster *et al.*, 2004). Death of the cell early after viral infection prevents the production of progeny, and such mechanisms would exert a strong impact on the slow growing betaherpesviruses. This evolutionarily ancient host defense strategy may be induced in mammals through intrinsic signals, as a consequence of cell stress induced by virus infection, or extrinsic signals through the engagement of death receptors on the cell surface or other immune effector mechanisms. Apoptotic bodies and cellular debris from dead cells are cleared by professional phagocytic cells, such as MΦ and DCs, which carry out critical roles priming the adaptive immune response. A wide range of cellular sensors may be triggered by viral infection, including alterations of the cellular tumor-suppressor p53 or other cell cycle regulators, alterations in mitochondrial function, nuclear changes resulting from DNA damage and repair, modification of endoplasmic reticulum, and

activation of PKR (Everett and McFadden, 1999, 2001). A wide range of antiapoptotic proteins encoded by DNA and RNA viruses have been recognized (Cuconati and White, 2002; Polster *et al.*, 2004), and many target the mitochondrion (Boya *et al.*, 2004). These inhibitors suppress cell death resulting from the intrinsic impact of virus infection or from extrinsic inducers or stimuli that accompany the host immune response. The consequence of keeping cells alive is enhancement of viral replication levels that increase the chances of a virus gaining a foothold in the host.

Betaherpesviruses rely on a variety of virus-encoded regulators that prevent cells from showing molecular or morphological hallmarks of apoptosis (Allart *et al.*, 2002; Brune *et al.*, 2003; Goldmacher *et al.*, 1999; Reboredo *et al.*, 2004; Skaletskaya *et al.*, 2001), as depicted in Fig. 21.1. Two HCMV genes, UL37x1 (Goldmacher *et al.*, 1999; Reboredo *et al.*, 2004), which encodes the viral mitochondrial localized inhibitor of apoptosis (vMIA), and UL36 (Skaletskaya *et al.*, 2001), which encodes the viral inhibitor of caspase 8 activation (vICA), inhibit apoptosis through clearly defined mechanisms. The major IE gene products have also been suspected of blocking apoptosis but little mechanistic insights have been gained. Four MCMV gene products inhibit apoptosis, with one (M36) being homologous to HCMV UL36 (McCormick *et al.*, 2003a; Menard *et al.*, 2003), one (m38.5) being a positional and functional homolog of vMIA (McCormick, 2005) and two (m41 and M45) functioning differently than any known HCMV gene product (Brune *et al.*, 2001, 2003; Hahn *et al.*, 2002; Patrone *et al.*, 2003). HHV-6B infected CD4+ cultures include apoptotic cells and virus-positive cells are more resistant to apoptosis than neighboring cells (Inoue *et al.*, 1997), suggesting that this virus also encoded genes that are antiapoptotic. The homologue of UL36 is the most likely candidate. There is also evidence that apoptosis may occur following exposure of non-permissive cells to betaherpes-viruses or in non-infected cells in productively infected cultures. Thus, all evidence is consistent with a role for betaherpesvirus gene products regulating apoptosis to prolong the life of the infected cell or make it resistant to host immune defense mechanisms. A role for suppression of apoptosis in species specificity of MCMV replication has also been demonstrated by converting human cells to a susceptible state using HCMV vMIA (Jurak and Brune, 2006).

vMIA (pUL37x1)

The genomic region of HCMV encoding the ORFs UL36, UL37, and UL38 is transcriptionally complex (Fig. 21.2). Sequence analysis combined with transcription and in vitro translation studies (Kouzarides *et al.*, 1988; Tenney and Colberg-Poley, 1990, 1991a, 1991b) indicated the presence of two immediate–early transcripts that include UL37x1. The larger transcript, 3.2–3.4 kb in length, is present only at immediate early times, encodes the glycoprotein gpUL37, and terminates at a polyadenylation signal located between UL36 and UL35. The more abundant, 1.7kb transcript, encoding pUL37x1, is present at immediate early times as well as throughout the remainder of infection. This transcript terminates at a polyadenylation signal located between UL38 and UL37x2. A splice variant encoding gpUL37$_M$ includes UL37x1, UL37x2, and a portion of UL37x3 (Goldmacher *et al.*, 1999). Transcription and translation in vitro predicted apparent molecular weights of 58 kDa and 24 kDa, respectively for gpUL37 and pUL37x1 (Tenney and Colberg-Poley, 1990). More recently, cDNA cloning has revealed that transcription through this region may produce as many as 11 spliced and unspliced transcripts (Adair *et al.*, 2003), including all those that had been previously identified. HCMV-induced alterations to the cellular splicing machinery apparently ensures the continued production of the unspliced transcript encoding pUL37x1 throughout infection (Su *et al.*, 2003).

The fact that UL37x1-containing gene products provide antiapoptotic activity emerged from transient expression of viral DNA fragments and functional analyses of pUL37x1, gpUL37 and gpUL37M clones in a cell death suppression assay (Goldmacher *et al.*, 1999). The name vMIA is reserved for the most potent of these, pUL37x1. vMIA prevents cell death induced by TNF, Fas ligand (Goldmacher *et al.*, 1999), TRAIL (Skaletskaya *et al.*, 2001), E1B19K deficient adenovirus (Goldmacher *et al.*, 1999), HIV Vpr (Roumier *et al.*, 2002), doxorubicin (Goldmacher *et al.*, 1999), nitric oxide, peroxynitrite, 4-hydroxynonenal (Vieira *et al.*, 2001), hydroxychloroquine (Boya *et al.*, 2003a), ionidamine, arsenite, the retinoid derivative CD437 (Belzacq *et al.*, 2001), propionibacterial short chain fatty acids (Jan *et al.*, 2002), *N*-(4-hydroxyphenyl)retinamide (Boya *et al.*, 2003b), and macroautophagy (Boya, 2005). vMIA increases the susceptibility of human cells to MCMV productive infection (Jurak and Brune, 2006). vMIA mediates protection at the level of the mitochondria and prevents release of cytochrome c and subsequent downstream events (Fig. 21.1), but does not prevent upstream events including cleavage of either procaspase-8 or Bid (Goldmacher, 1999). vMIA can be immunoprecipitated with adenine nucleotide transporter, a component of the mitochondria membrane pore that interacts with Bax and other Bcl-2 family members but for vMIA, this interaction is non-specific. vMIA also interacts with Bax in cells (Arnoult *et al.*, 2004; Poncet *et al.*, 2004), and Bax also localizes to mitochondria during MCMV infection (Andoniou *et al.*, 2004). More recently an interaction with growth arrest and DNA damage 45 alpha (GADD45α) was established

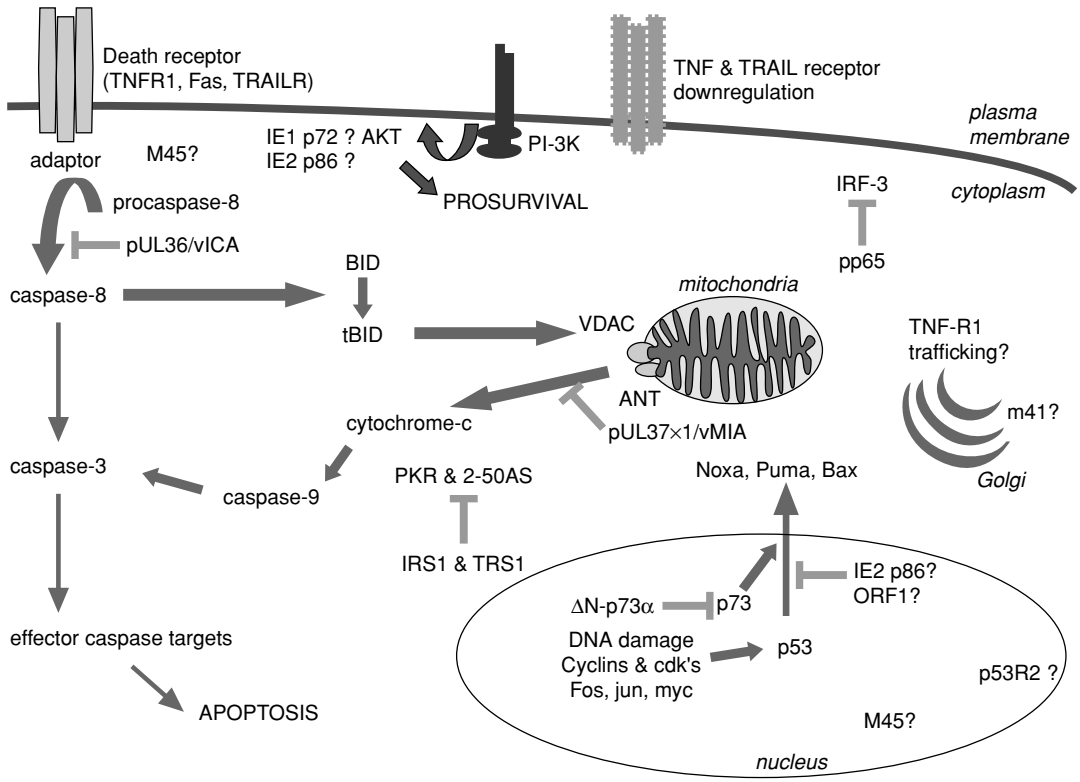
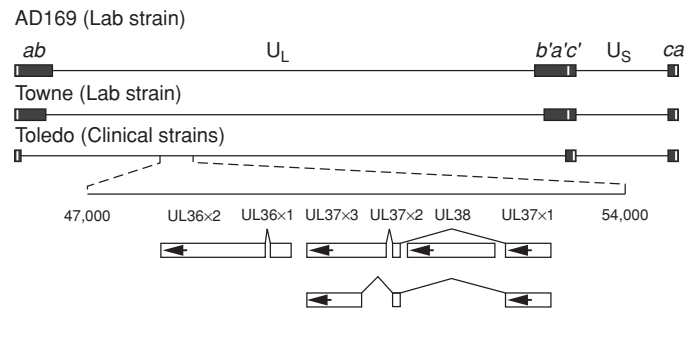


Fig. 21.1. Betaherpesvirus inhibition of apoptosis and interferon response. Grey arrows represent proapoptotic pathways while black arrows indicate prosurvival pathways and black lines indicate interruption of proapoptotic pathways. Cellular functions are listed in grey font and viral functions are indicated in black font. Plasma membrane, cytoplasm, nucleus, mitochondria, and Golgi, are depicted in italics. Bold black text indicates viral inhibitors with known or suspected mechanisms. Question marks indicate possible points of interference by viral proteins.

through yeast two hybrid and significantly, this interaction was shown to be critical for cell-death protection since addition of any GADD45 family member (alpha, beta, or gamma) enhanced survival and function was impaired by siRNA-mediated GADD45 family reduction. vMIA lacks the BH domains that characterize Bcl-2 family members and Bax also localizes to mitochondria during MCMV infection (Andoniou *et al.*, 2004). Rather this, protein is composed of an amino terminal region, aa 5–34, that is important for localization to mitochondria and a carboxyl terminal region, aa 118–147, that is critical for anti-apoptotic activity (Hayajneh *et al.*, 2001). This domain also mediates the interaction with GADD45 family members that facilitates vMIA activity (Smith *et al.*, 2005).

vMIA does not exhibit sequence variation in HCMV (Hayajneh *et al.*, 2001) and sequence homologs can only be found in primate CMVs. The regions of highest sequence conservation are coincident with regions defined by mutational studies to be required for vMIA activity (McCormick *et al.*, 2003b). "In fact, a minimal 69 aa protein that includes amino acids 1–34 and 112–147 of vMIA retains full activity (Hayajneh *et al.*, 2001) and is very similar to homologues in monkey CMVs (McCormick *et al.*, 2003b)." At a positionally conserved location in the viral genome, MCMV and rat CMV retain a functional homolog which despite limited sequence homology, functions in cell death assays (McCormick *et al.*, 2005). A UL37x1 homolog has not been identified in other betaherpesviruses, although all betaherpesviruses carry a gene homologous to UL37x3, which as the largest exon, has been annotated as UL37 in most betaherpesviruses but that lacks independent anti-apoptotic activity (Chapter 15; McCormick *et al.*, 2003b). UL37x1 is not essential for viral replication in Towne*var*ATCC, a strain carrying a functional vICA (McCormick *et al.*, 2005). In contrast, transposon mutants disrupting HCMV UL37x1 fail to produce infectious virus, in AD169*var*ATCC, a strain carrying a mutant UL36 (Reboredo *et al.*, 2004). Other, independently derived UL37x1 deletion mutants made in AD169*var*ATCC had also suggested that vMIA is required for viral replication (Brune *et al.*, 2003; Dunn *et al.*, 2003; Yu *et al.*, 2003). Thus, vMIA is dispensable unless other mutations are present which render the virus



	vICA	gpUL37	gpUL37$_M$	vMIA
AD169*var*ATCC	–	+	+	+
AD169*var*DE	+	+	+	+
Towne*var*ATCC	+	+	+	+
Towne*var*RIT	–	–	–	+
Toledo passage 8	+	+	+	+

Fig. 21.2. Presence of functional antiapoptotic genes in HCMV strains. (top) Depiction of the commonly used laboratory strains AD169, Towne, and Toledo genomes. Rectangles represent repeated *ab* - *b′a′* sequence flanking the unique long (U_L) and the c′a - *ac* sequences flanking the short (U_S) genome components. An expansion of the region including nucleotides 47,000–54,000 is depicted with a representation of ORFs (open rectangles) in the UL36-UL37 region. Splicing patterns relevant to proteins with demonstrated antiapoptotic function are indicated by lines connecting ORFs and polyadenylation sites are indicated by arrowheads. The presence (+) or absence (–) of vICA, gpUL37, gpUL37$_M$, and vMIA in AD169*var* ATCC, AD169*var* DE, Towne*var* ATCC, Towne*var* RIT and Toledo (passage 8) is indicated below the ORF diagram (Dunn *et al.*, 2003; Skaletskaya *et al.*, 2001).

dependent on the gene to prevent apoptosis. (Dunn *et al.*, 2003; Brune *et al.*, 2003; Reboredo *et al.*, 2004; Yu *et al.*, 2003; Skaletskaya *et al.*, 2001). Consistent with this conclusion and in contrast to the caspase-dependent cell death noted for strains that require vMIA, premature death in Towne*var*ATCC mutant virus is caspase-independent (McCormick *et al.*, 2005).

vICA (pUL36)

This betaherpesvirus-conserved anti-apoptotic gene product is dispensable for HCMV replication in cultured fibroblasts and is mutated in many common laboratory strains (Patterson and Shenk, 1999; Skaletskaya *et al.*, 2001), as depicted in Fig. 21.2. Evidence that UL36 encodes the anti-apoptotic protein vICA first emerged from transient expression of viral DNA fragments in a cell death suppression assay (Skaletskaya *et al.*, 2001). Caspase 8, the target of pUL36, is also known as FLICE. vICA is mechanistically similar to the viral and cellular FLICE inhibitory proteins (v-FLIP and c-FLIP), but lacks any sequence similar to death effector domains typical of this class of protein. Like FLIPs, vICA prevents cleavage by binding to the pro-domain of procaspase-8 and provides protection from apoptosis initiated by death receptors TNFR, TRAILR, or Fas that require caspase 8 activation. vICA only slightly delays cell death induced by E1B19K deficient adenovirus or doxorubicin. The presence of vICA correlates with an increased resistance of HCMV to inducers of extrinsic cell death (Skaletskaya *et al.*, 2001). Mutants of the MCMV homologue M36 show a reduced growth phenotype in macrophages (IC-21, J774-A1, and peritoneal exudate cells) but not in fibroblasts or endothelial cells, but this behavior does not appear to extend to the HCMV gene product (Dunn *et al.*, 2003). M36 mutant infected cells are, however, more susceptible to induction of apoptosis by the Fas pathway similar to HCMV viruses defective in UL36 (Skaletskaya *et al.*, 2001).

UL36 homologs and vICA function are widely conserved among betaherpesviruses and sequence conservation includes regions outside the boundaries of the US22-family domains (McCormick *et al.*, 2003a). The homolog is an immediate early gene in MCMV, while the rhesus macaque CMV homolog is an early gene similar to vMIA in that virus. The homologs of UL36 in HHV-6A and HHV-7 are each encoded by a spliced transcript. The HHV-6A is regulated as an immediate–early gene (Flebbe-Rehwaldt *et al.*, 2000), whereas the HHV-7 spliced RNA seems to be regulated as an early gene (Menegazzi *et al.*, 1999).

Other cell death suppressors

Ribonucleotide reductases convert ribonucleoside diphosphates to deoxyribonucleoside diphosphates and are generally important for DNA synthesis and repair. While alphaherpesviruses and gammaherpesviruses encode both ribonucleotide subunits RR1 and RR2 and make an active enzyme, the betaherpesviruses only encode a homolog of the RR1 subunit that lacks enzymatic activity (Chapter 15). Viral mutants of MCMV M45, but not the HCMV homolog UL45, show cell-type dependent growth properties (Brune *et al.*, 2001; Hahn *et al.*, 2002). Insertional mutants of M45 grow similar to wild-type viruses in cultured fibroblasts, bone marrow stromal cells, and hepatocytes but fail to grow in either endothelial cells or macrophages (Brune *et al.*, 2001), which enter apoptosis by about 1 day post-infection. Cell to cell spread is thus severely restricted. The alphaherpesvirus HSV-2 RR1 subunit prevents cell death upstream of caspase 8 activation due to an amino-terminal extention relative to other RR1 homologs (Langelier *et al.*,

2002) in addition to being a component of an active ribonucleoside reductase enzyme. It is possible that M45 has preserved such an antiapoptotic function, although there is little sequence similarity to guide how the two may be related. Cell-type restricted growth and induced apoptosis are not observed in HCMV UL45 mutants, which grow poorly and are less efficient at cell-to-cell spread (Patrone *et al.*, 2003), but do not exhibit cell type specific defects in fibroblasts, macrophages, or endothelial when used at high MOIs (Hahn *et al.*, 2002). Mutant HCMV is somewhat reduced compared to wt in ability to withstand Fas-induced apoptosis (26% survival vs. 50% survival), however; HCMV UL45 is unable to independently block cell death in the absence of viral infection.

MCMV mutant defective in m41 prematurely kills cells and replicates to reduced levels compared to parental virus (Brune *et al.*, 2003). Caspase inhibition reduces but does not eliminate cell death, suggesting that apoptosis may underlie the process. Expression of epitope-tagged m41 during viral infection shows localization to Golgi. The mechanism of protection remains to be elucidated.

HCMV IE1 p72 and IE2 p86 are nuclear proteins that regulate transcription of cellular and viral genes (Chapters 17 and 18) and each can associate with cellular proteins. Cellular transcription factor E2F is modulated by IE1 p72 interaction with the Rb-pocket protein p107 (Poma *et al.*, 1996) or by IE2 p86 interaction with pRB (Hagemeier *et al.*, 1994). Regulation of E2F1 is mediated by binding to IE1 p72 (Margolis *et al.*, 1995). IE1 p72 and IE2 p86 suppress apoptosis induced by TNF or E1B19K defective adenovirus (Zhu *et al.*, 1995) and anti-apoptotic activity maps to disparate sequences without any hint of mechanism. Importantly, fibroblasts constitutively expressing IE1 p72 that have been used to complement growth of IE1 p72 mutant viruses do not exhibit any obvious altered cell cycle progression or susceptibility to apoptosis. In transient assays, a genomic clone including IE1 p72 and IE2 p86 rescues a temperature sensitive derivative of $TAF_{II}250$ mutant cells (*ts* 13) from transcriptional repression and apoptosis but not cell cycle arrest (Lukac *et al.*, 1997). Further analysis indicated either IE2 p86 or IE1 p72 activates the PI3 kinase pathway and AKT as a consequence (Yu and Alwine, 2002) similar to the response of these prosurvival pathways to infection (Johnson *et al.*, 2001b), but the role of IE1 p72 and IE2 p86 as suggested by the $TAF_{II}250$ mutant cells has not been extended to natural infection. IE2 p86 but not IE1 p72 protects from overexpression of p53 in smooth muscle cells (Tanaka *et al.*, 1999). In contrast IE2 p86 but not IE1 p72 expression in endothelial cells induces apoptosis (Wang *et al.*, 2000). Control of cell-intrinsic responses is the suggested mechanism for HCMV IE1 facilitation of MCMV replication in human cells (Tang and Maul, 2006). These data may suggest that any potential protective role includes both an induction-specific and a cell-type specific component.

Alteration of extrinsic cell death pathways during infection

TNF-R1 surface expression levels decrease following HCMV infection of macrophage or astrocytoma cell lines (Baillie *et al.*, 2003) as well as following MCMV infection of bone marrow derived macrophages (Popkin and Virgin, 2003). Although the viral factors required for the down-regulation have not been identified, either virus seems to employ a post-transcriptional mechanism. Uninfected fibroblasts insensitive to TRAIL mediated apoptosis become sensitive to TRAIL following HCMV infection (Sedger *et al.*, 1999). Infection increases expression of death-inducing TRAIL-R1 and TRAIL-R2, but not decoy receptors TRAIL-R3 and TRAIL-R4. Analysis of HHV-7 revealed downregulation of TRAIL-R1 but not TRAIL-R2, TNF-R1, TNF-R2, or Fas during infection of CD4 cells (Secchiero *et al.*, 2001). Infections by other betaherpesviruses maintain the Fas receptor available for activation. Fibroblasts infected by HCMV and MCMV are susceptible to Fas-mediated apoptosis (Chaudhuri *et al.*, 1999; Goldmacher *et al.*, 1999; Menard *et al.*, 2003; Skaletskaya *et al.*, 2001) and some, but not all, strains of HCMV may increase Fas receptor surface expression (Chaudhuri *et al.*, 1999; Chiou *et al.*, 2001).

HCMV encodes a potential TNF-receptor homolog (Benedict *et al.*, 1999), although this protein has not been assigned any role in blocking apoptosis. UL144 encodes a glycoprotein consisting of a leader peptide, cysteine-rich domains (CRD), membrane extension region, transmembrane domain, and a short cytoplasmic tail. UL144 exhibits dramatic strain-to-strain sequence variability (Bale, 2001; Lurain, 1999), although the protein has highly conserved transmembrane and cytoplasmic domains. UL144 is the closest known relative of the cell surface herpesvirus entry mediator (HVEM; Chapter 7) protein, whose normal function is as a cognate ligand for the B- and T-lymphocyte attenuator (Sedy *et al.*, 2005). UL144 binds B and T lymphocyte attenuator (BTLA) and inhibits T-cell proliferation.

Summary

The betaherpesviruses all appear to alter cell cycle and to block rather than promote apoptosis during infection. Cytomegaloviruses prevent cellular DNA synthesis whereas roseolaviruses allow continued cellular DNA

synthesis during productive infection, although cell division is blocked in all of these viruses. While none of the betaherpesviruses has been implicated in malignancy, such an impact on the host cell has raised interest of persistent betaherpesvirus infection in certain chronic diseases. These viruses encode a wide variety of functions that modulate the cellular environment, including functions that modulate cell cycle progression and that derail apoptosis induced by either intrinsic or extrinsic mediators.

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Part II

Basic virology and viral gene effects on host cell functions: gammaherpesviruses

Edited by Patrick Moore

22

Introduction to the human γ-herpesviruses

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Introduction

This chapter will provide a brief background into the γ-herpesviruses family in comparison to other members of the herpesvirus family; but the primary focus of this chapter will be to recount the discovery of the two human γ-herpesviruses (EBV and KSHV) and the diseases associated with infection of each virus, a brief introduction into their life cycles, and finally a description of the genome characteristics of the viruses including a description of their respective genomes. In many ways, the discovery and association with human diseases for both EBV and KSHV have many parallels despite almost three decades separating their discoveries and association with human disease.

The γ-herpesvirus family

The γ-herpesviruses are a subfamily of herpesviruses that were first distinguished by their cellular tropism for lymphocytes. Subsequent molecular phylogenetic analyses have confirmed the close relationship among these viruses that is distinct from the α- and β-herpesviruses subfamilies (Fig. 22.1). *Gammaherpesvirinae* is currently divided into two genera, *Lymphocryptoviridae* which includes human Epstein–Barr virus (EBV or HHV4) and *Rhadinoviridae*, which includes human Kaposi's sarcoma-associated herpesviru*s* (KSHV or HHV8). Recent studies suggest that primate rhadinoviruses can be further subdivided in KSHV-like viruses, a second closely related but distinct lineage of Old World primate viruses related to the rhesus rhadinovirus (RRV), and the New World monkey rhadinoviruses represented by herpesvirus saimiri (HVS). A more detailed analysis of the non-human γ-herpesviruses will be discussed in Chapters 60 and 61.

Although the best-studied members of the γ-herpesviruses are EBV and KSHV, γ-herpesviruses are parasites of a broad range of mammals from mice (murine herpesvirus-68 and related viruses) to cows and horses (bovine herpesvirus 4 and equine herpesvirus 2), as well as primates. Surprisingly, γ-herpesviruses of lower mammals most closely resemble the rhadinoviruses, and exhibit extensive molecular piracy of host regulatory genes that is not found in EBV and related viruses. Interestingly, the lymphocryptoviruses have been found only in primates and humans. The γ-herpesviruses share a similar genomic structure that the 172 kilobase pair, linear double-stranded DNA genome of the B95–8 EBV strain serves as the prototype since it was the first γ-herpesvirus that was sequenced (Baer *et al.*, 1984). The large central region of the genome contains most of the coding capacity for the viruses, including blocks of highly conserved genes that are shared among the herpesviruses (Fig. 22.2). The ends of the molecule are capped by variable numbers of direct repeat sequences that are the sites for genome circularization during latency. Unlike the α-herpesviruses (Chapter 10), the γ-herpesviruses do not undergo genomic isomerization and only linearize and recircularize in their terminal repeat regions, although the numbers of terminal repeats can be highly variable.

The γ-herpesviruses also share a number of characteristics in common with the α- and β-herpesviruses, particularly related to lytic viral replication. During lytic replication, the γ-herpesviruses genome is packaged as a linear molecule in a proteinaceous capsid, which is then enveloped by a lipid bilayer prior to release from the cell. This process starts when viral transactivators initiate viral genome-wide transcription through a series of orderly transcriptional cascades. Different classes of viral genes (immediate–early, early and late) are transcribed resulting in the production of infectious virions. As with other

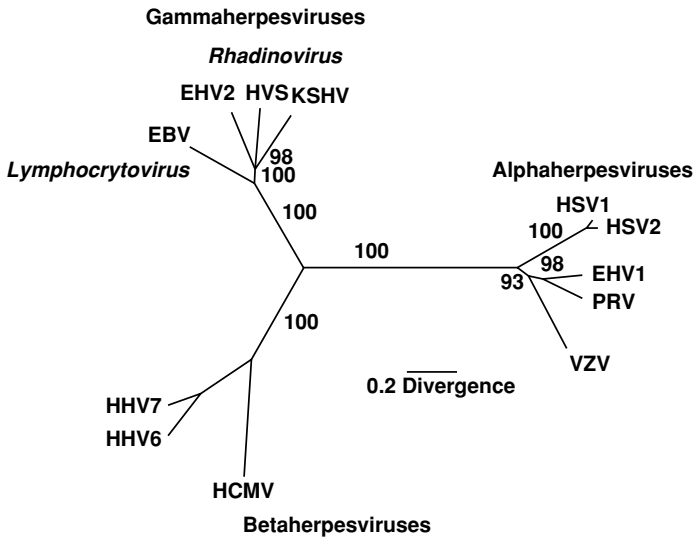

Fig. 22.1. Phylogenetic tree for selected herpesviruses. Phylogenetic trees are based on comparison of aligned amino acid sequences between herpesviruses for the MCP gene. The comparison of MCP sequences was obtained by the NJ method and is shown in unrooted form, with branch lengths proportional to divergence (mean number of substitution events per site) between the nodes bounding each branch. The number of times (of 100 bootstrap samplings) that the division indicated by each internal branch was obtained is shown next to each branch; bootstrap values below 75 are not shown. Figure adapted from Fig. 2A from reference (Moore *et al.*, 1996). Used with permission of American Society of Microbiology.

herpesviruses, γ-herpesviruses lytic replication is thought generally to cause cell death. While specific mechanisms for lytic replication differ between the herpesviruses, and even among the γ-herpesviruses, they all broadly share similar capsid structures and have similar overall mechanisms for lytic replication. As would be expected, genes involved in lytic replication and viral capsid production tend to be highly conserved across the herpesvirus subfamilies, including the γ-herpesviruses.

The γ-herpesviruses differ the most dramatically from each other and from other herpesviruses during the latent portion of their lifecycle. Unlike the other human herpesviruses, both EBV and KSHV latency can be established and manipulated in vitro, providing an important experimental system that is unavailable for other viruses. Latency occurs after infection of a cell and transport of the capsid to the nucleus where the genome is released. The viral genome, still a linear DNA fragment, then circularizes by ligation of terminal repeat sequences and replicates as an episome using the host cell replication machinery.

A key feature of the γ-herpesviruses is their common capacity to induce lymphoproliferation and cancers. Tumors caused by EBV and KSHV include lymphoproliferative diseases and lymphomas, but also include tumors from other tissue-types, such as smooth muscle cells and endothelial cell origin. In some cases, tumorigenesis occurs when viruses cross species such as bovine infection by the wildebeest γ-herpesviruses, alcelaphine herpesvirus 1, in Africa (Ensser *et al.*, 1997). In other cases, tumorigenesis is a rare occurrence among infected individuals, except when the host is specifically susceptible through immunosuppression or through a rare familial mutation altering normal immune function.

EBV was the first human tumor virus discovered and has been a rich source of information for tumor biologists as well as a tool for immortalizing cell lines for use as reagents. As shown by the Henles in 1967, EBV has the unusual property of immortalizing primary B lymphocytes in culture (Henle *et al.*, 1967). Continuous cell lines that result from this immortalization are termed lymphoblastoid cell lines (LCLs) reflecting their activated phenotype. These LCLs contain EBV episomes and express a very limited number of viral genes and have served as an important model of EBV latent infection and transformation as will be further discussed in Chapter 24. These latently infected cell lines, as shown in 1978 by zur Hausen and colleagues can be induced to lytic replication by treatment with phorbol esters (zur Hausen *et al.*, 1978). Interestingly, similar treatment of cell lines harboring KSHV causes induction of lytic replication (Arvanitakis *et al.*, 1996; Renne *et al.*, 1996).

KSHV, more recently discovered, provides a unique tumor virus model since it has incorporated cellular proto-oncogenes and serves as a rosetta stone between cancer biology and tumor virology. The natural lifecycles of EBV and KSHV are well studied and experimentally tractable allowing for the careful examination of oncogenes from these viruses not only in terms of their capacity to induce cell transformation but also in terms of their roles in the natural viral life cycle.

The discovery of Epstein–Barr virus (EBV)

Although there was considerable evidence to indicate the role of viruses in human cancers from animal studies dating from the early 1900s, it was not until the identification of Burkitt's lymphoma and the subsequent identification of EBV in this unusual tumor that a clear role of viruses in human cancers became apparent. The first key to the puzzle of a human virus being associated with a human

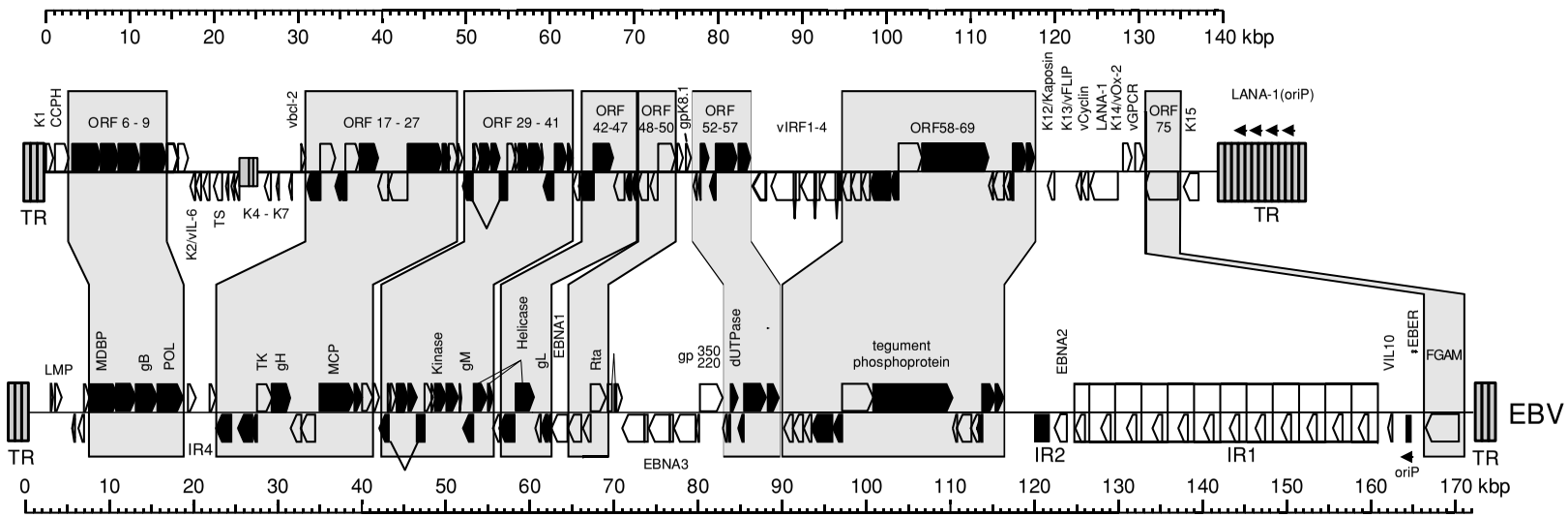

Fig. 22.2. Colinearity of the genetic maps of KSHV (top) and EBV (bottom). Schematic representation of the genomes of KSHV and EBV were drawn to scale based on the sequences by Russo *et al.* (PNAS 93, 14862–14867, 1996; Genbank acc. no. U75698) and the inverted sequence of EBV strain B95–8 (Baer *et al.*, *Nature* **310** (5974), 207–211 (1984), Genbank acc. no. NC001345), respectively. According to Russo *et al.*, protein coding regions (open reading frames, ORF) of KSHV that are conserved amongst KSHV and herpesvirus saimiri are numbered from 1 to 75. KSHV ORFS without detectable homology to herpesvirus saimiri genes are numbered with the prefix "K" or from K1 to K15. Open arrows symbolize herpesvirus genes not conserved amongst all human herpesvirus groups (alpha, beta, and gamma), whereas "core" herpesvirus genes that share homology detectable by BLAST amongst all groups of human herpesviruses are represented by arrows filled in black. The names of a few hallmark-genes are given above the EBV genome. Essentially, conserved genes are localized in five (seven) major blocks on the genomes of KSHV and EBV, linked here by trapezoid boxes. Most genes of the lytic replication cycle (virion proteins, DNA-replication proteins) are found here. These blocks of core herpesvirus genes are interspersed by areas coding for more strain- or virus-specific genes. In the case of KSHV, these are usually the "K"-genes that are frequently homologous to genes of the host cell. In the case of EBV, the Epstein-Barr virus nuclear antigens (EBNA 1–3) and latent membrane proteins (LMP1–2) are present at these positions. In addition, two major envelope glycoproteins of KSHV and EBV, gpK8.1 and gp350–220, respectively, are not conserved amongst the viruses. However, both genes are located at comparable positions close to the center of the coding regions.

Abbreviations: CCPH, complement control protein homologue; MDBP, major DNA binding protein; gB, glycoprotein B; pol, DNA polymerase; vIL-6, viral IL-6 homologue; vbcl-2, viral bcl-2; TK, thymidine kinase; gH, glycoprotein H; MCP, major capsid protein; gM, glycoprotein M; gL, glycoprotein L; Rta: R-transactivator of transcription; vIRF1–4: interferon regulatory factors 1–4; vFLIP: viral flice-inhibitory protein; vCyclin: viral cyclin-D homologue; vOx-2: viral Ox-2 (CD200) homologue; LANA-1: latent nuclear antigen 1; vGPCR: viral G-protein coupled receptor homologue; FGAM: formyl-glycin-amid transferase homologue; vIL10: viral interleukin 10 homologue; EBER: EBV-encoded small RNAs; TR, terminal repeat; IR: internal repeat; oriP: plasmid origin of replication.

tumor resulted from the interest of Denis Burkitt with a malignancy in children in Africa.

Denis Burkitt was born in 1911 and lived in Lawnkilla near Enniskillen, County Fermanagh, Ireland. He received his medical training at Trinity College in Dublin with his clinical training at Adelaide Hospital. Following graduation and after working in hospitals in his native Ireland and military service as army surgeon in the Royal Army Medical Corps, Burkitt embarked on a career of medical service in Africa due in part to his strong religious convictions and interest in service in the third world. In 1957, after being in Kampala, Uganda, for 10 years, he was asked by Hugh Trowell, a colleague in Kampala, to see a young boy with swellings on both sides of his upper and lower jaws that proved to be a lymphoma. The tumor was a very common cancer in African children, was fast growing causing grotesque disfigurement, and was fatal upon metastasis to other parts of the body.

Burkitt's fascination and interest in the tumor led him to carefully examine hospital records and to send out a questionnaire to government and mission hospitals throughout Africa. He also embarked on what he would call his long safari to document the incidence of this lymphoma. From this trip, which took 10 weeks, covered 10 thousand miles, journeyed through 12 countries, and stopped at 57 hospitals; Burkitt found a definite pattern of distribution with the lymphoma confined to a region 10 degrees north and 10 degrees south of the equator following closely the pattern of distribution of mosquito borne diseases such as malaria and yellow fever. These initial results were published in the *British Journal of Surgery* in 1958 (Burkitt, 1958). But it was not until 1961 after publication of a more detailed version in *Cancer* with a pathologist Greg O'Conor (Burkitt and O'Conor, 1961) and an invitation to give a lecture at Middlesex Hospital in London in March of 1961 that allowed the next step in the identification of EBV to fall into place.

In the audience of Burkitt's talk at Middlesex Hospital was Anthony Epstein who immediately developed an interest in the Burkitt's tumor. Epstein, who had worked on the Rous sarcoma virus, had developed an interest in the role of viruses in cancer and he was fascinated with Burkitt's description of his findings. Rous sarcoma virus was shown in 1911 by Peyton Rous at the Rockefeller Institute in New York to be responsible for a sarcoma that was transmissible in chickens. Epstein and Burkitt immediately began to collaborate. Biopsies were flown from Kampala to Epstein's laboratory in London, but it was not until 1963 after Yvonne Barr and Bert Achong joined the hunt that herpesvirus-like particles were observed in February 1964 from a cell line established from a biopsy delivered from Africa early in December of 1963 (Epstein *et al.*, 1964). Interestingly, both Achong and Barr were graduates of schools in Ireland. Achong graduated from University College Dublin and Yvonne Barr was a graduate of Trinity College Dublin.

Human disease associated with EBV infection

Considerable interest has focused on EBV since its discovery and its link with Burkitt's lymphoma. Along with KSHV, as we will learn also in this chapter, EBV is the only other herpesvirus with an etiological role in human malignancies. As described above, EBV is a causative agent in endemic Burkitt's lymphoma, but since the link of EBV with this lymphoma, EBV has also been shown to be important for a large number of additional diseases in humans.

Shortly after the description of EBV association with Burkitt's lymphoma, the Henles, a husband and wife team at the Children's Hospital of Philadelphia obtained a set of cell cultures from Epstein. Werner and Gertrude Henle were both born and educated in Germany. Werner emigrated to the United States in 1936 finding an instructorship position at the University of Pennsylvania. Werner's grandfather, Jacob Henle, was of Jewish descent, making his further training and livelihood in question because of the increased power of the Nazi regime in Germany and the approaching war. It had become "increasingly clear" to Werner that he could "not stay in Germany and pursue a career to his liking." In 1937, Gertrude Szpingier, who was Werner's fiancée, joined Werner in Philadelphia. They had met at the University of Heidelberg and were married the day after Gertrude's arrival in the United States. They spent their entire careers at the University of Pennsylvania making not only important discoveries in regard to EBV, but also other aspects of virology, immunology, and viral oncology.

With the cultures in hand from Epstein, the Henles began to explore the presence of antibodies directed against the new virus. By analyzing the immune response, Gertrude and Werner demonstrated that the EBV was widespread in the human population (Henle and Henle 1966; Henle *et al.*, 1969). As expected, they found antibodies to EBV in children with Burkitt's lymphoma, but also in the serum of healthy African children. Also, as may have been expected, the antibody titers in Burkitt's lymphoma patients were much higher that in healthy children. More surprising, was the finding that antibodies were found in most serums from children tested all over the world indicating that the virus was ubiquitous within the human population. Burkitt's lymphoma cell lines in culture were also used at this time by Lloyd Old and his colleagues at the Memorial Sloan–Kettering Cancer Center in New York to make the initial

connection of EBV infection and nasopharyngeal carcinoma (Old *et al.*, 1966).

Because antibodies to EBV were so ubiquitious, the Henles suspected that infections with EBV were common and were generally self-limited in nature. Interestingly, this has been a common observation in regard to most of the herpesviruses that were subsequently discovered. At the time of the discovery of EBV, only three other human herpesviruses were known. These were herpes simplex virus (HSV), varicella-zoster virus (VZV), and cytomegalovirus (CMV). All of these viruses caused overt symptoms as in the case for HSV and VZV, or were associated with serious congenital defects. As will be discussed in other chapters and this chapter, HHV6, HHV7, and KSHV cause little disease in immune competent individuals. It is only when the immune host immune system is compromised that overt symptoms typically appear with infection of these viruses.

As is the case for many discoveries, serendipity led to the discovery of the association of EBV with infectious mononucleosis. Late in 1967, a technician working in the Henle laboratory developed classical symptoms of infectious mononucleosis (Henle *et al.*, 1968). Prior to her symptoms, she had shown no antibodies to EBV, but with her symptoms, antibodies appeared to EBV. As will be reviewed in the later chapters, we will learn that, along with the association of EBV with Burkitt's lymphoma, NPC, and infectious mononucleosis, EBV has now been found to be involved in a much wider range of human disease. It is generally accepted that EBV is involved with several other malignancies of lymphocyte origin such as some types of Hodgkin's lymphoma, and epithelial origin such as gastric carcinoma. Table 22.1 contains a list of diseases with known EBV etiology. Each of these disease associations will be expanded in later chapters and specific references can be found in these chapters. EBV is also a factor in a variety of other human malignancies including some T and NK cell lymphomas.

The association of EBV with other diseases, such as breast carcinoma and hepatocellular carcinoma, remain controversial and will likely only be resolved in the coming years with additional research. In immunosuppressed patients, EBV causes a variety of proliferative disorders including oral hairy leukoplakia in AIDS patients, immunoblastic lymphomas, and an unusual tumor of muscle origin in children with AIDS or who are under immune suppression for liver transplantation. In young boys with X-linked immunodefiencies, EBV causes severe mononucleosis that results in death.

Evidence is accumulating that the EBV may also be associated with immune mediated diseases. In particular, there is a variety of auto-immune diseases that appear to have an infectious agent as a cofactor such as multiple sclerosis, rheumatoid arthritis, and diabetes. For each of these autoimmune diseases, it has been suggested that EBV may have an involvement in causing deregulation of the normal immune response to self-antigens. But, since EBV is so ubiquitous, many of these studies are also controversial. The linking of EBV infection to autoimmune disease and controversial malignancies such as breast carcinoma and hepatocellular carcinoma may await the development of an effective vaccine against EBV which will be discussed in Chapter 72. By preventing primary infection, an effective vaccine would offer convincing proof of a disease association due to absence of a particular disease or malignancy in those vaccinated for EBV. This may allow the true appreciation of the wide variety of disease associated with EBV infection in humans.

EBV life cycle

Infection with EBV usually occurs early in childhood, resulting in an asymptomatic infection. The virus is spread through saliva. If primary infection occurs later, B-cell proliferation and the resulting immune response results in infectious mononucleosis. After primary infection, most individuals will harbor the virus for the remainder of their life, and carriers develop cellular immunity against a variety of both lytic and latency associated proteins that will be more discussed later in this chapter as well as in Chapter 51. By adulthood, the majority of the human population (upwards of 90%) is infected with EBV. Periodically, virus is shed from latently infected individuals by the induction of lytic replication in B lymphocytes. The true site of latent infection has not been determined, but the virus likely resides in B lymphocytes. Recent studies have shown that EBV can be detected in circulating peripheral blood lymphocytes in carriers of EBV latent infections by PCR (both for viral DNA and viral mRNA) (Tierney *et al.*, 1994; Chen *et al.*, 1995; Babcock *et al.*, 1999; Qu *et al.*, 2000) and virus isolation and outgrowth of immortalized cell lines by culturing peripheral lymphocytes (Yao *et al.*, 1985). It has not been determined if this is the true site of latency. Other potential sites of EBV latency may include bone marrow, lymph nodes, or other lymphoid organs.

Early experiments suggested that latency is not maintained by constant re-infection of circulating B-lymphocytes as evidenced in patients treated with acyclovir (Yao *et al.*, 1989). Acyclovir, a nucleoside analogue that can inhibit lytic replication in the oral epithelium had no effect on the number of B-cells in the peripheral blood population that harbor the virus. More recent experiments have shown

Table 22.1. EBV associated pathologies in the human host

Pathology	Sub-type	Percent EBV +	Notes
Burkitt's lymphoma			
	Endemic	95–100%	Africa and New Guinea
	Sporadic	20–30%	Outside endemic region
Infectious mononucleosis		100%	
Chronic active EBV infection		100%	
Nasopharyngeal carcinoma		100%	Near 100% association with Type II and Type III, Type I frequently associated with EBV in endemic regions (South East Asia and North Africa)
Hodgkin's disease			90% in children in Latin America
	Mixed cell, lymphocyte depleted	60–80%	
	Nodular schlerozing	20–40%	
Fatal IM/X-linked lymphoproliferative syndrome		100%	
Immunocompromised disorders			
	Post-transplant Lymphoproliferative disorder	80%	
	Burkitt's lymphoma	30–50%	
	Diffuse large cell lymphoma – Centroblastic	30%	
	Diffuse large cell lymphoma – Immunoblastic	90%	
	Primary CNS lymphoma	100%	
	Non-Hodgkin's lymphoma	30–50%	
	Leiomyosarcoma	100%	Unusual muscle tumor found primarily in children
	Oral Hairy leukoplakia	100%	
T-Cell lymphoma			
	Nasal T/NK lymphomas	100%	EBV Infected B cells detected along with the lymphoma. T/NK cells can be infected with EBV.
	Angioimmunoblastic lymphadenopathy with dysproteinemia	30%	
Gastic carcinoma			
	Undifferentiated carcinoma of nasopharyngeal type	100%	
	adenocarcinoma	5–15%	

that viral gene expression may be greater than may have previously been thought in lymphoid organs such as the tonsil suggesting that the virus may manipulate normal B-cell development and survival to insure continued latency by the expansion of infected cells without lytic replication (Miyashita *et al.*, 1997; Babcock *et al.*, 1998, 2000; Babcock and Thorley-Lawson, 2000; Joseph *et al.*, 2000a,b; Thorley-Lawson, 2001; Hochberg *et al.*, 2004a,b). This will be more fully discussed in later chapters.

Further evidence of the hematopoetic site of EBV latency comes from engraftment of bone marrow cells that can result in the loss of the resident virus or the appearance of a new virus strain from donor lymphocytes (Gratama *et al.*, 1989). Lytic replication is presumed to occur when EBV infected B lymphocytes traffic through and transmit infection to oral epithelium providing a source for infection of other individuals. Interestingly, recent studies have suggested different virus strains are present within different compartments in humans with EBV latent infection such as peripheral blood and the oral cavity suggesting that epithelial infection may be more important than previously thought (Sitki-Green *et al.*, 2003). Understanding the complex interplay in EBV latency in the human host and the importance of viral gene expression requires the careful

analysis of EBV gene function which will be the topic of other chapters.

The discovery of Kaposi's sarcoma-associated herpesvirus (KSHV)

The discovery of KSHV or human herpesvirus 8 (HHV8), has many parallels with the discovery of Epstein–Barr virus. Like Dennis Burkitt, Patrick Moore and Yuan Chang, were intrigued by the appearance of a new cancer in the United States, but rather than appearing in very young children, the disorder was appearing in young healthy gay men (Jaffe *et al.*, 1983). This disorder was Kaposi's sarcoma (KS). KS was originally described as idiopathic purplish pigmented sarcoma of the skin by Moriz Kaposi in 1872 (Kaposi, 1872). Kaposi, born Moriz Kohn in Kaposvar, Hungary, obtained his MD in Vienna in 1861 and worked with Ferdinand Hebra, the founder of a renowned School of Viennese Dermatology. He would later become Hebra's son-in-law in 1869 and his successor in 1881. Also in 1869, Kaposi wrote the initial description of lupus erythematosus with a more comprehensive description published in 1872. He also described xeroderma pigmentosum. In 1871, two years after converting from Judaism to Roman Catholic and marrying Hebra's daughter, he changed his name to reflect his birthplace. In contrast to the frequent surname Kohn, Kaposi was certainly unique in Vienna, thus avoiding being mixed-up with others. In addition, this name change may have guarded against the harsh anti-semitism during the rule of Emperor Franz-Josef. Like his more comprehensive description of lupus erythematosus, it was also in 1872 that Kaposi published his description of the sarcoma that now bears his name. KS was a relatively rare, indolent, pigmented growth typically found on the skin of elderly men. Initially, Kaposi reported on the clinical features of six cases, including one of a young boy, but the remaining cases were all in men over 40 years of age. He reported that the disease was incurable and often resulted in death within two years after the disease appeared. In the 1960s, before the AIDS epidemic, it was realized that KS was not as rare as initially thought. The incidence in equatorial tropical Africa or sub-Sarahan African is much greater as will be more fully discussed below.

Following the outbreak of AIDS in the 1980s, in which there was a dramatic increase of KS in AIDS patients, there was considerable interest in the further study of this unusual malignancy and in particular the association of KS with infection with a human pathogen. Like Burkitt's lymphoma, a role for a virus was suspected in KS lesions long before the onset of HIV infection and the resulting AIDS epidemic. As early as 1972, herpesvirus-like particles were found in electron microscopic analysis of KS biopsies (Giraldo *et al.*, 1972). This virus was determined to be cytomegalovirus (CMV), a herpesvirus that is ubiquitous in the human population. By the early 1990s, a number of other pathogens had also been found in KS lesions by a variety of investigators. Typically, these agents were not found in all the lesions and in many cases they were ubiquitous agents in the human population and were subsequently dismissed as having a role in KS. Like Burkitt's lymphoma, there was epidemiological data that suggested the role of an infectious agent in the development of KSHV. But before the identification of KSHV, it was thought that HIV may be the critical factor in the development of AIDS-associated KS. However, the uneven distribution of KS among different transmission groups for the human immunodeficiency virus (HIV) resulted in the hypothesis that an environmental factor or a transmissible agent other than HIV was involved in KS pathogenesis (Beral *et al.*, 1990). Most notably, whereas more than 20% of homosexual and bisexual AIDS patients developed KS, only 1% of age- and sex-matched men with hemophilia suffered from this uncommon tumor, suggesting transmission of a KS-related virus by sexual practice.

The first real break that led to the discovery that a herpesvirus was linked to KS came in 1993 when Yuan Chang moved to Columbia University to take up a position in neuropathology. Chang received her medical degree from the University of Utah College of Medicine. Patrick Moore married Yuan Chang and in 1989 he left his job at the Centers for Disease Control to follow Chang to New York. Moore received a MS from Stanford in 1980, his MD from the University of Utah School of Medicine in 1985, and his MPH from the University of California, Berkeley in 1990. Once in New York, Moore was unable to find an appropriate academic position, so he joined the New York City Health Department. Both Moore and Chang were interested in identifying new pathogens without in vitro culture since this was a question that Moore was interested in from his work at the Centers for Disease Control. A recent publication from Michael Wiglers' laboratory at Cold Spring Harbor detailed a new technique called representational difference analysis (RDA). This technique, which was developed by the Lisitsyns, used PCR to identify DNA sequences present in one sample but not a control sample. On the surface, this looked ideal for the identification of unique infectious agents. The paper describing this technique was published in *Science* (Lisitsyn *et al.*, 1993). It was not used to detect unique pathogen, but only the feasibility of using this technique was demonstrated using lambda or adenovirus DNA. Barry Miller, a colleague of Patrick Moore at the Centers for Disease Control, suggested that Moore and Chang apply this technique in their pathogen discovery.

With a single KSHV lesion and control tissue, the husband and wife team began to perform RDA. The initial experiments were performed by Chang and Melissa Pessin. Pessin was a pathology resident on a research rotation at Columbia. Moore helped in the evenings after finishing his duties at the New York City Health Department. The initial amplifications resulted in four prominent bands. Two KS lesions were positive for two of the four bands by Southern hybridization, but surprisingly, a control tissue from an AIDS patient was also positive. The control tissue was from an unusual lymphoma found in AIDS patients. This lymphoma, characterized by pleural, pericardial, or peritoneal lymphomatous effusions, is referred to as primary effusion lymphoma (PEL) or body-cavity based lymphoma and would also be shown to be positive for the new herpesvirus that Chang and Moore identified as will be detailed later.

Testing a larger panel tissue, Moore and Chang found that virtually all of the KS lesions were positive while none of the controls showed the same number of positives. From sequencing the RDA products, they developed internal PCR primers for one of the sequences that allowed a simple PCR based screen for analysis of additional tissues. Surprisingly, when the initial sequences were compared to sequences available in the current databases, no homologous sequences were identified. This suggested an unknown pathogen. Moore quit the Health Department in January 1994 so that he could devote his efforts full-time to the project at Columbia. Chang made an important contact working in the spring of 1994 with Frank Lee and Janice Culpepper at the DNAX Institute for molecular biology. Lee, working with newly developed BLAST sequence alignment algorithms, was able to show that DNA fragments isolated by RDA from KS lesions unambiguously belonged to a new herpesvirus, similar to but distinct from known herpesviruses. With this data in hand, a paper was submitted to *Science* and was accepted for publication in December of 1994, identifying a new herpesvirus (Chang *et al.*, 1994). Both Moore and Chang have recently moved to the University of Pittsburgh School of Medicine, where Chang is a Professor of Pathology and Moore is a Professor of Molecular Genetics and Biochemistry and Director of the Molecular Virology Program at the University of Pittsburgh Cancer Institute.

KSHV life cycle

Like EBV, KSHV requires intimate contact for transmission. At least in regions endemic for KS, all evidence points to similar modes of transmission in young children as has been described for EBV (Mayama *et al.*, 1998; Martro *et al.*, 2004). Primary infection likely occurs from contact via saliva with parents, siblings, playmates, and close relatives at a very early age. Similarly, in adult populations close intimate contact is also required. Interestingly, in comparison to EBV the infection rates of KSHV within the human population can vary dramatically throughout the world population based on serology. In endemic regions such as Africa, infections rates are well over 50%, whereas in Northern Europe and the United States, infection rates range from 1–6% (Gao *et al.*, 1996; Kedes *et al.*, 1996; Simpson *et al.*, 1996). Southern Europe, which like Africa, has a higher incidence of KS, also has a higher rate ranging from 10%–30% when compared to infection rates in the United States and Northern Europe (Schatz *et al.*, 2001). As might be expected, infection rates measured by serology are higher in homosexual men than in the general population in western countries, ranging from 20%–40% in homosexual men not suffering from KS (Martin *et al.*, 1998). In immunocompromised KSHV infected individuals, a wide assortment of cells and tissues has been shown to harbor KSHV. Most frequently, peripheral B cells have been reported to carry the KSHV genome (Ambroziak *et al.*, 1995). But T-cells, monocytes, and endothelial cells have also been found positive for KSHV DNA (Henry *et al.*, 1999; Blackbourn *et al.*, 2000). In contrast, the site of latency in immunocompetent individuals is essentially unknown. Difficulties in detecting viral DNA in seropositive, healthy individuals may reflect a low frequency of spontaneous KSHV reactivation, which would in turn result in the scarceness of KSHV positive cells and relatively low KSHV transmission rates at least in North America, Northern Europe and most parts of Asia. As a consequence, sites of persistence and the overall strategy of KSHV latency in the human host have been difficult to determine as of this date. The supposedly low rate of spontaneous reactivation in immunocompetent individuals is also reflected by low and declining antibody titers (Martro *et al.*, 2004). The latter has made it difficult initially to estimate the seroprevalence of KSHV in the general population (Pellett *et al.*, 2003), as will be detailed in Chapter 54.

Human disease associated with KSHV infection

Symptoms related to primary infection with KSHV have only recently been described (Andreoni *et al.*, 2002). Like EBV, it would appear that symptoms in immunocompetent individuals are very modest with primary infection being largely asymptomatic. In children, this primary infection may be associated with a febrile maculopapular skin rash (Andreoni *et al.*, 2002). Reports have indicated that primary infection in adults undergoing immune suppression for organ transplantation have experienced bone marrow failure, splenomegaly, and fever (Luppi *et al.*, 2002). There

is also evidence that KSHV may be transmitted by solid organ transplants, eventually resulting in the development of rapidly progressing KS (Barozzi *et al.*, 2003; Marcelin *et al.*, 2004). However, at present it is not clear whether screening of organ donors for KSHV infection is beneficial.

Despite the only recent identification of KSHV, remarkable progress has been made in the identification of pathological consequences of infection with KSHV. Along with EBV, as described above, KSHV is also associated with proliferative disorders in both immune competent and immune deficient humans. In endemic regions of Africa, KS is a common debilitating cancer among men, women, and children. Following the establishment of this link, KSHV has also been shown to be important for a number of other diseases in the human population as will be described.

Classical KS, as originally identified by Kaposi, was typically seen in elderly Mediterranean patients, was indolent in nature, and affected the skin of the lower limbs. In endemic KSHV infection in Africa, before the HIV epidemic hit, KSHV infection presents as four distinct clinical syndromes: relatively benign, nodular cutaneous disease which is very similar to classical KS, aggressive cutaneous disease which invades both bone and localized soft tissue, florid visceral and mucocutaneous disease, and finally lymphadenopathic disease that can rapidly disseminate to lymph nodes and visceral organs (quite often in the absence of cutaneous disease). The final syndrome typically occurs in children. KS associated with immune suppression either from HIV infection or organ transplantation commonly presents multifocally and symmetrically and may arise quickly. These lesions will often undergo spontaneous remission with improving immune status.

As discussed earlier, there were early indications that another proliferative disorder observed in HIV patients might also be related to KSHV infection. Early work by Chang and Moore found that an unusual lymphoma in patients with AIDS was also positive for KSHV DNA (Chang *et al.*, 1994). The lymphoma was confined primarily to body cavities and grows as an effusion. Hence the names primary effusion lymphoma (PEL) or body-cavity based lymphoma. Daniel Knowles and Ethel Ceserman working with Moore and Chang established that these tumors are also uniformly positive for KSHV (Cesarman *et al.*, 1995). Interestingly, the vast majority of these tumors are also positive for EBV.

The final disease in human associated with KSHV infection is multicentric Castleman's disease (MCD). MCD was first described by Castleman in 1956 and is an unusual lymphoproliferative disorder that is characterized by lymphadenopathy, fever, and splenic infiltration (Castleman *et al.*, 1956). KSHV is present in nearly all the cases of MCD in AIDS patients as originally shown by Soulier and colleagues in 1995 and about half of the cases in HIV-negative patients (Soulier *et al.*, 1995). MCD is considered to be a semi-malignant lymphoproliferative disorder, associated with IL-6 hyperproduction and inflammatory symptoms. In the context of MCS, however, a highly aggressive plasmablastic non-Hodgkin-lymphoma may arise which is also KSHV positive and likely to have arisen from monoclonal "microlymphomas" detectable in some MCD lesions (Dupin *et al.*, 2000).

Finally, there have been reports of the potential association of KSHV infection with sarcoidosis (Di Alberti *et al.*, 1997) and multiple myeloma (Rettig *et al.*, 1997), but as is the case with EBV association with liver and breast cancer, the linkage of KSHV infection with these two pathologies requires additional confirmation before a definitive link is established. In 2003, C. D. Cool *et al.* detected DNA and antigen of KSHV in lung tissues of 10 out of 16 patients with severe primary pulmonary hypertension (Cool *et al.*, 2003). It is fascinating to note that, like MCD, PEL, and KS, primary pulmonary hypertension is frequently associated with HIV-1 infection. However, several follow-up studies were not able to confirm this intriguing finding (Henke-Gendo *et al.*, 2004 and reviewed in D. Rimar *et al.*, July 2006). But C. D. Cool and coworkers argue, that in contrast to their initial work most follow-up studies were based on serological assays. In fact, only two of the studies used DNA-PCR and/or histochemistry Katano *et al.*, 2005; Daibata *et al.*, 2004), and these were performed on patients from Japan, were KSHV prevalence is very low. Thus, further work is needed before a link between PPH and KSHV infection can be considered shown.

Phylogenetic relationship between EBV, KSHV, and non-human γ-herpesvirus genomes

The γ-herpesviruses are split into two subfamilies: the γ1-herpesviruses and the γ2-herpesviruses. The two human γ-herpesviruses include the recently identified KSHV, and EBV, which are distinguished by their latent infection of lymphoblastoid cell lines either of T- or B-cell origin. EBV is the only human member of the genus γ1-herpesvirus also termed lymphocryptovirus. A number of related viruses have been identified that infect both New World and Old World primates (Wang, 2001; Wang *et al.*, 2001). These viruses serve as important models to investigate the pathogenesis of the lymphocryptoviruses in vivo (see Chapters 60 and 61). KSHV is a member of the γ2-herpesviruses or rhadinoviridae and like EBV has important related viruses that infect New and Old World primates. Herpesvirus saimiri (HVS), which infects squirrel monkeys, has been well studied and also provides an important model of KSHV in vivo pathogenesis (Fickenscher and Fleckenstein, 2001).

Following the discovery of KSHV in man, several studies were undertaken to detect additional Old-World primate rhadinoviruses. By searching for antibodies cross-reactive with KSHV, the group of Ronald Desrosiers at the New England Primate Research Centre isolated the rhesus monkey rhadinovirus (RRV) (Desrosiers *et al.*, 1997). The complete genomic sequence of RRV clearly showed that this virus is more closely related to KSHV than herpesvirus saimiri (Searles *et al.*, 1999). In particular, most genes not conserved between KSHV and herpesvirus saimiri, the so-called "K" genes, do have homologues in RRV. In contrast to HHV-8, RRV can be readily propagated in cell culture. While RRV was discovered at the East Coast, Timothy M. Rose and Marnix Bosch were sleepless in Seattle. They studied tissue specimens from rhesus monkeys suffering from retroperitoneal fibromatosis (RF) at the Washington Regional Primate Research Centre. RF has been identified as an infrequent disease syndrome occurring in immunosuppressed macaques (Giddens *et al.*, 1985). RF lesions somewhat resemble KS: they consist of an aggressively proliferating fibrous tissue with a high degree of vascularization, and transmission studies indicated that an infectious agent may be involved in RF pathogenesis. By using a degenerated PCR technique, fragments of a herpesvirus DNA polymerase gene were identified in RF tissues from two macaque species, *Macaca nemestrina* and *Macaca mulatta* (Rose *et al.*, 1997). Sequence comparisons indicated that, at least the DNA polymerase genes of these two novel rhadinoviruses, tentatively termed RFHVMm and RFHVMn, are more closely related to KSHV than the DNA polymerase genes of RRV. In addition, the LANA gene of RVHVmn is closely related to KSHV in structure and sequence. A role of RFHVMn in the pathogenesis of RF is suggested by the finding that RF spindle cells are highly positive for RFHVMn LANA (Burnside *et al.*, 2006). However, attempts to isolate these viruses on cultured cells have not been successful so far. Several rhadinoviruses have been discovered since then in various Old World primates, including chimpanzees, gorillas and orang-utans. Phylogenetic analysis clearly showed that these Old Word primate rhadinoviruses form two clades. KSHV is the prototype of one clade, and RRV the prototype of the second clade. The thrilling point here is: most Old World primate species seem to harbor representatives of both clades. The search for a second rhadinovirus in man has not been successful to date, however.

Human γ-herpesviruses genomes

At least two EBV types have been identified in human populations and these were formerly designated EBV type A and type B (Zimber *et al.*, 1986) but have recently been designated EBV-1 and EBV-2 to parallel the HSV-1 and HSV-2 nomenclature. The majority of EBV isolates in western communities are type 1, while type 2 EBV isolates appear to be largely restricted to equatorial Africa and Papua New Guinea. Unlike HSV-1 and HSV-2, there is extensive nucleotide homology and restriction endonuclease site conservation throughout most of the EBV-1 and EBV-2 genomes. However, there are important differences in the sequence of key EBV genes such as EBNA2 and the EBNA3 family of genes (Dambaugh *et al.*, 1984; Sample *et al.*, 1990). But, overall, the two types of EBV are considerably more closely related to each other than are HSV-1 and HSV-2 (Lees *et al.*, 1993). Related to the differences in key EBV genes, the EBV-1 strains transform lymphocytes more readily and to faster growing cell lines than do EBV-2 strains (Rickinson *et al.*, 1987).

EBV has a linear double-stranded genome of approximately 172 kilobase pairs (kb) and has a base composition of 59% guanine plus cytosine. It was the first large DNA virus whose complete sequence was determined (Baer *et al.*, 1984). Subsequent to the sequencing of the EBV genome, the VZV, HSV, CMV, HHV6, and KSHV genomes were cloned and sequenced. The EBV genome sequenced, designated B95–8, was from an EBV-infected marmoset cell line partially permissive for lytic replication (Miller *et al.*, 1972). Upon further analysis, it was determined that this virus isolate contained a deletion of approximately 12 kb relative to other EBV strains. Sequence analysis of Raji EBV strain revealed the 12 kb region deleted from B95–8 contained three potential open reading frames (Parker *et al.*, 1990). The sequence analysis to date predicts around 85 to 95 open reading frames (Table 22.3, Fig. 22.2). The nomenclature for each identified gene in the sequence is based on the BamHI fragment in which the reading begins. This is followed by an "L" or an "R," depending on the whether the reading frame is leftward or rightward. The reading frames in each BamHI fragment are then numbered. As is apparent from the Table 22.2, many of the reading frames also have another name based on the prior identification of the gene product or homology to well described genes or gene products described in other herpesviruses.

The KSHV genome is a linear, double-stranded DNA of approximately 160 kbp and has the overall structure typical for rhadinoviruses (Fig. 22.2) (Russo *et al.*, 1996). A complete rhadinovirus genome is usually termed M genome, as it is of intermediate density (M-DNA). The γ2-herpesviruses were termed "rhadino" viruses utilizing the ancient greek word for fragile, because this M-DNA tends to split into two fractions of DNA molecules with highly different density, the L-DNA containing genes (low density, low G+C content) and the terminal repetitive H-DNA (high density, high G+C content). The latter is, as far as

Table 22.2. KSHV associated pathologies in the human host

Pathology	Sub-type	Percent KSHV Positive	Notes
Kaposi's sarcoma	Classical	100%	
	African endemic	100%	
	iatrogenic immunosuppression	100%	
	AIDS-associated	100%	
Primary effusion lymphoma		~100%	50% of PEL are also positive for EBV
Multifocal Castleman disease	HIV-associated	90%–100%	
	Non-HIV associated	~40%	
	Plasma cell variant	50–100%	50% in HIV-negative, up to 100% in HIV positive patients
	Hyaline vascular variant	Usually negative	
Primary pulmonary hypertension		60%	unconfirmed report

known until today, without coding capacity. The L-DNA contains at least 89 open reading frames, 67 of which have homologues in the closely related γ2-herpesvirus prototype herpesvirus saimiri. The overall amino acid sequence identity of these 67 KSHV reading frames to homologues identified in herpesvirus saimiri ranges from 22.4 to 66% (average: 42%). Conserved genes are usually found in a comparable genomic position and orientation. Thus, KSHV genes that share homology with herpesvirus saimiri are numbered from left to right according to their position on the herpesvirus saimiri genome. Open reading frames which do not share recognizable amino acid homology with genes in herpesvirus saimiri are numbered with the prefix "K" (Table 22.4). Until today, 19 genes have been identified which are not clearly homologous to genes identified in herpesvirus saimiri (K1 – K15, K4.1, K4.2, K8.1, K10.5). Frequently, these "K" genes are strikingly homologous to known cellular genes. They code for proteins interfering with the immune system, for enzymes of the nucleotide metabolism, and for putative regulators of cell growth (Table 22.4).

Despite enormous differences in base composition, it is apparent that herpesvirus genomes have many open reading frames in common. Many products of EBV genes can be predicted on the basis of their amino acid homology with HSV genes and genes from other herpesvirus genes such as KSHV. The predictions and known functions are shown in Tables 22.3 and 22.4. These similarities between proteins encoded by the different herpesviruses, such as HSV-1, KSHV, and EBV underscores the relationships between the various herpesviruses. The homologous genes are primarily limited to genes required for the cleavage and packaging of the viral genome and infection of susceptible host cells. Included among the conserved genes are virion structural proteins, enzymes involved in DNA replication, some regulators of gene expression, and glycoprotein genes. Lytic gene function has been primarily described in HSV, because lytic replication is easily observed in tissue culture systems allowing gene function studies. In contrast, EBV and KSHV, which are largely latent in tissue culture systems, have been less amenable to studies of lytic gene function.

Characteristics of the γ-herpesvirus virion

Morphologically, the EBV and KSHV virions are very similar to other herpesvirus virions, consisting of an envelope containing viral glycoproteins (Epstein *et al.*, 1964, 1965). A tegument layer is found between envelope and nucleocapsid. The envelope contains viral transmembrane glycoproteins that mediate attachment and entry, either via fusion or endocytosis. Most glycoproteins of EBV and KSHV are conserved amongst the different herpesvirus families (gB, gH, gL, gM and gN). However, the most abundant envelope glycoproteins of EBV and KSHV are not found in more distant herpesviruses. These are termed gp350/220 and gpK8.1, respectively (Hutt-Fletcher, 1995; Raab *et al.*, 1998). The EBV and KSHV capsids are similar to those of other herpesviruses. The icosahedral capsid shells are composed of 162 capsomers (12 pentons, 150 hexons). It contains the linear, double stranded DNA molecule wrapped around a toroid-like protein core. The three-dimensional structure of the KSHV capsid has been determined by cryo electron-microscopy using viral particles produced from cultured primary effusion lymphoma (PEL) cells at a resolution of 22 – 24A (Wu *et al.*, 2000).

Conclusions

There has been dramatic progress in the understanding the molecular biology and disease associations of oncogenic

Table 22.3. EBV genes

EBV gene	HSV gene	KSHV gene	EBV Name	Known or proposed function
BNRF1		ORF75		Tegument protein, putative phosphoribosylformylglycinamidine synthase
EBER1,2				*Cell survial factor*
BCRF1				IL-10 homologue – host immune modulator
BCRF2				
BWRF1			*EBNALP*	*Regulator of latent viral gene Transcription (EBNA5)*
BYRF1			*EBNA2*	*Major regulator of viral gene Transcription*
BHRF1		ORF16		Bcl-2 homologue
BHLF1				
BFLF2	UL31	ORF69		Associates with nuclear matrix
BFLF1	UL32	ORF68		Virion protein – DNA cleavage/packaging
BFRF1	UL34	ORF67		Virion protein – capsid assembly
BFRF2		ORF66		
BFRF3	UL35	ORF65		Capsid protein
BPLF1	UL36	ORF64		Virion phosphoprotein – DNA release
BOLF1	UL37	ORF63		Cytoplasmic phosphoprotein
BORF1	UL38	ORF62		Capsid assembly – binds DNA
BORF2	UL39	ORF61		Ribonucleotide reductase (large subunit)
BaRF1	UL40	ORF60		Ribonucleotide reductase (small subunit)
BMRF1	UL42	ORF59		Polymerase associated processivity factor
BMRF2	*UL43*	ORF58		Transmembrane glycoprotein, 53/55kDa
BSLF2/ BMLF1	UL54	ORF57		Post-transcriptional regulator of viral gene expression
BSLF1	UL52	ORF56		DNA replication – helicase/primase complex
BSRF1	*UL51*	ORF55		
BLRF1		ORF53		gN
BLRF2		ORF52		p23 capsid antigen
BLLF1a			gp350	Virion binding to CR2 (CD21)
BLLF1b			gp220	Virion binding to CR2 (CD21)
BLLF3	UL50	ORF54		dUTPase
BLRF3-BERF1		*EBNA3C*		*Regulator of latent viral gene Transcription (EBNA6)*
BERF2a,b		*EBNA3B*		*Regulator of latent viral gene Transcription (EBNA4)*
BERF3-BERF4		*EBNA3A*		*Regulator of latent viral gene Transcription (EBNA3)*
BZLF2			gp42	Complexes with gp25 and gp85 – binds HLA Class II
BZLF1				Z transactivator
RAZ				Z regulator
BRLF1		ORF50		R transactivator
BRRF1		ORF49		Transcription factor
BRRF2		ORF48		
BKRF1			*EBNA1*	*Maintenance of viral episome*
BRKF2	UL1	ORF47	gp25	gL – complexes with gp42 and gp85
BKRF3	UL2	ORF46		Uracil-DNA glycosylase
BKRF4	*UL3*	ORF45		Nuclear phosphoprotein
BBLF4	UL5	ORF44		DNA replication – helicase/primase complex
BBRF1	UL6	ORF43		Virion protein – DNA packaging
BBRF2	UL7	ORF42		
BBLF3	UL8	ORF41		DNA replication – helicase/primase complex
BBLF2	UL9	ORF40		DNA replication – helicase
BBRF3	UL10	ORF39		gM
BBLF1	UL11	ORF38		Myristylated virion protein
BGLF5	UL12	ORF37		exonuclease
BGLF4	UL13	ORF36		Virion protein kinase

Table 22.3. (*cont.*)

EBV gene	HSV gene	KSHV gene	EBV Name	Known or proposed function
BGLF3	UL14	ORF34		Putative virion tegument protein, anti-apoptotic
BGLF3.5		ORF35		
BGRF1	UL15	ORF29a		DNA packaging protein
BGLF2	UL16	ORF33		Capsid maturation/assembly protein
BGLF1	UL17	ORF32		Capsid maturation/DNA packaging
BDLF4		ORF31		
BDRF1	UL15	ORF29b		DNA packaging protein
BDLF3		*ORF28*	gp150	Enhances epithelial infection
BDLF3.5		*ORF30*		
BDLF2		ORF27		
BDLF1	UL18	ORF26		Minor capsid protein
BcLF1	UL19	ORF25		Major capsid protein
BcRF1		ORF24		
BTRF1	*UL21*	ORF23		
BXLF2	UL22	ORF22	gp85	gH – complexes with gp25 and gp42
BXLF1	UL23	ORF21		Thymidine kinase
BXRF1	UL24	ORF20		Membrane protein – fusion
BVRF1	UL25	ORF19		Virion protein
BVRF2	UL26	ORF17		Serine protease
BILF2			gp78/55	
BILF1				GPCR
BALF5	UL30	ORF9		DNA polymerase
BALF4	UL27	ORF8	gp110	gB – virus maturation
BALF3	UL28	ORF7		DNA cleavage/packaging
BALF2	UL29	ORF6		ssDNA binding protein
BALF1				
BARF0/RK-BARF0				*Regulator of notch pathway*
BARTS				*Various functions?*
BARF1				Growth factor
BNLF1a,b,c			*LMP1*	*Constitutive CD40 mimic – oncoprotein*
			LMP2A	*Constitutive B Cell receptor mimic (TP1)*
			LMP2B	*Regulator of LMP2A and LMP1 function? (TP2)*
Raji LF3				
Raji LF2		ORF11		
Raji LF1				Part of BILF1

Note: Genes in italics in the "EBV" column are expressed in EBV latent infections. In the "HSV" and "KSHV" columns, the homology of the genes in italics with EBV is statistically not significant when standard blast comparisons are made. However, due to positional analogy, it is likely that the genes are evolutionary related. Based on the sequence data published in references (Baer *et al.*, 1984; McGeoch *et al.*, 1985, 1988; Parker *et al.*, 1990; Russo *et al.*, 1996).

γ-herpesviruses like EBV and KSHV. Many years of research on lymphomas induced by EBV and herpesvirus saimiri have identified viral factors that are critical for the proliferative disorders observed with these viruses. Upon infection of naïve B- or T-cells, respectively, differentiation and proliferation are induced in an antigen-independent manner, most likely to expand the pool of virus -infected or -infectable cells. To achieve this goal, both EBV and herpesvirus saimiri make use of transmembrane proteins mimicking constitutively active signaling receptors. In the case of EBV, the latent membrane proteins 1 and 2A are likely essential for the differentiation and expansion of latently infected cells observed in vivo. In herpesvirus saimiri, these latent transmembrane proteins are STP (saimiri transforming protein) and a second protein termed TIP (tyrosine kinase interacting protein) that is found in some strains of herpesvirus saimiri. Usually, this process does not result in the development of malignant disease, but is likely important for the establishment of latency. However, other circumstances can occur which may result

Table 22.4. KHSV genes

KSHV gene	HSV gene	EBV gene	KHSV name	Known or proposed properties and function
K1			K1	Transmembrane glycoprotein, signaling protein
ORF04			CCPH	Complement binding protein
ORF06	UL29	BALF2		ssDNA binding protein
ORF07	UL28	BALF3		DNA cleavage/packaging
ORF08	UL27	BALF4		gB – virus maturation
ORF09	UL30	BALF5		DNA polymerase
ORF10				
ORF11		Raji LF2		
K2			*vIL-6*	*viral interleukin-6, direct binding to gp130*
ORF02			DHFR	Dihydrofolate reductase
K3			IE-1B	Downregulates MHC I
ORF70				Thymidylate synthase
K4			vMIP-II / vMIP-1a	Viral macrophage inflammatory protein (chemokine)
K4.1			vMIP-III / vMIP-1b	Viral macrophage inflammatory protein (chemokine)
K4.2				
K5			IE-1A	Downregulates MHC I
K6			vMIP-I / vMIP-1a	Viral macrophage inflammatory protein (chemokine)
K7				partially overlaps w. non-translated T1.1RNA (=Nut-1, PAN RNA)
ORF16		BHRF1		Bcl-2 homologue
ORF17	UL26	BVRF2		Serine protease
ORF18				
ORF19	UL25	BVRF1		Virion protein
ORF20	UL24	BXRF1		Membrane protein – fusion
ORF21	UL23	BXLF1		Thymidine kinase
ORF22	UL22	BXLF2		gH – complexes with gp25 and gp42
ORF23	*UL21*	BTRF1		
ORF24		BcRF1		
ORF25	UL19	BcLF1		Major capsid protein
ORF26	UL18	BDLF1		Minor capsid protein
ORF27		BDLF2		
ORF28		*BDLF3*		
ORF29a	UL15	BGRF1		DNA packaging protein
ORF29b	UL15	BDRF1		DNA packaging protein
ORF30		*BDLF3.5*		
ORF31		BDLF4		
ORF32	UL17	BGLF1		Capsid maturation/DNA packaging
ORF33	UL16	BGLF2		Capsid maturation/assembly protein
ORF34	UL14	BGLF3		Putative virion tegument protein, anti-apoptotic
ORF35		BGLF3.5		
ORF36	UL13	BGLF4		Virion protein kinase
ORF37	UL12	BGLF5		Exonuclease
ORF38	UL11	BBLF1		Myristylated virion protein
ORF39	UL10	BBRF3		gM
ORF40	UL9	BBLF2		DNA replication – helicase
ORF41	UL8	BBLF3		DNA replication – helicase/primase complex
ORF42	UL7	BBRF2		
ORF43	UL6	BBRF1		Virion protein – DNA packaging
ORF44	UL5	BBLF4		DNA replication – helicase/primase complex
ORF45	UL3	BKRF4		Nuclear phosphoprotein
ORF46	UL2	BKRF3		Uracil-DNA glycosylase

Table 22.4. (*cont.*)

KSHV gene	HSV gene	EBV gene	KHSV name	Known or proposed properties and function
ORF47	UL1	BRKF2		gL – complexes with gp42 and gp85
ORF48		BRRF2		
ORF49		BRRF1		Na transcription factor
ORF50		BRLF1	Rta, art, lyt-a	KSHV Rta transactivator
K8			K-bZip, RAP	Represses Rta, required for viral DNA replication
K8.1			gpK8.1, gp35–57	Viral envelope glycoprotein, antigenic, binds to heparan sulfate
ORF52		BLRF2		p23 capsid antigen
ORF53		BLRF1		gN
ORF54	UL50	BLLF3		dUTPase
ORF55	*UL51*	BSRF1		
ORF56	UL52	BSLF1		DNA replication – helicase/primase complex
ORF57	UL54	BSLF2/ BMLF1		Transactivator/repressor
K9			vIRF-1	Viral interferon regulatory factor
K10			vIRF-4	Viral interferon regulatory factor
K10.5			*vIRF-3, LANA-2*	*Viral interferon regulatory factor, latent nuclear Antigen 2*
K11			vIRF-2	Viral interferon regulatory factor
ORF58	*UL43*	BMRF2		Putative integral membrane glycoprotein
ORF59	UL42	BMRF1		Polymerase associated processivity factor
ORF60	UL40	BaRF1		Ribonucleotide reductase (small subunit)
ORF61	UL39	BORF2		Ribonucleotide reductase (large subunit)
ORF62	UL38	BORF1		Capsid assembly – binds DNA
ORF63	UL37	BOLF1		Cytoplasmic phosphoprotein
ORF64	UL36	BPLF1		Virion phosphoprotein – DNA release
ORF65	UL35	BFRF3		Capsid protein
ORF66		BFRF2		
ORF67	UL34	BFRF1		Virion protein – capsid assembly
ORF67.5	UL33	BFRF4		DNA packaging
ORF68	UL32	BFLF1		Virion protein – DNA cleavage/packaging
ORF69	UL31	BFLF2		Associates with nuclear matrix
K12			*Kaposin A,B,C*	
K13			*vFLIP*	*Anti-apoptotic viral flice inhibitory protein*
K14			*vCyclin*	*Cyclin-D homologue*
ORF73			*LANA*	*Latent nuclear antigen, maintenance of latent viral genome,* functional analogue to EBV EBNA 1
K14			vOx-2	Viral Ox-2 homologue, immunomodulatory protein
ORF74			vIL8R, vGPCR	viral interleukin-8 receptor, viral G-protein coupled receptor; constitutively active, KS-like lesions in transgenic mice
ORF75		BNRF1		Tegument protein, putative Phosphoribosylformylglycinamidine synthase
K15				Signaling protein

Note: Genes in italics in the "KSHV" column are expressed in KSHV latent infections. In the "HSV" and "EBV" columns, the homology of the genes in italics with KSHV is statistically not significant when standard blast comparisons are made. However, due to positional analogy, it is likely that the genes are evolutionary related. Based on sequence data published in references (McGeogh *et al.*, 1985, 1988; Parker *et al.*, 1990; Russo *et al.*, 1996).

in overt malignant disease such as accidental infection of a foreign host, immunosuppression, or infection with malaria. Each of these increases the risk of development of disease associated with viral infection. In addition, genetic changes may occur in infected cells within the host that may also be important for the development of malignant disease. This may be quite rapid as seen in Burkitt's lymphoma or have a lengthy latency period as seen in Hodgkin's lymphoma. Besides the latent membrane proteins, other EBV viral proteins such as EBNA1 (Wilson *et al.*, 1996; Drotar

et al., 2003) and the virally encoded small RNAs called the EBERs (Komano *et al.*, 1999; Ruf *et al.*, 2000) may also have a role in EBV-associated malignant diseases in the human host. This will be more fully covered in later chapters.

Several lines of evidence indicate that KSHV plays an essential role in KS pathogenesis: the virus is invariably present in KS, KSHV infection precedes KS-development, the virus is present in the tumor cells themselves, and it is rather infrequently found outside the population at risk of KS (see Chapters 50 and 54). Research on KSHV pathogenesis is hampered by the lack of a cell culture system for transformation and animal models for KSHV proliferative diseases. Studies using rodents transgenic for single KSHV genes have been more successful, but results from such experiments should still be interpreted with caution, no matter how tempting the resemblance of lesions in mice transgenic for vIL8R to KS may be (Yang *et al.*, 2000).

At least two concurring models exist for the role of KSHV in oncogenesis. The "cytokine model" emphasizes the role of inflammatory cytokines induced or produced by KSHV. A closer look at clinical course and histopathology of KS raises doubts about the relevance of "classical," transforming genes for the pathogenesis of this peculiar tumor. The peculiar pathology of KS hints at a more complex, indirect mechanism of pathogenesis. Based on clinical observations and data derived from cell culture systems, models of KS pathogenesis were developed before KSHV was discovered. Several groups agreed upon the notion that KS develops as an interplay of inflammatory cytokines and angiogenic factors (Ensoli and Stürzl, 1998), although the cytokines focused on varied in different reports. Interestingly, KSHV encodes or induces several cytokines with intriguing similarity to the cellular factors shown to be required for in vitro models of KS. An example is VEGF that is secreted by cells expressing the constitutively active vIL-8R. This leads to a model of KS pathogenesis, where increased secretion of both viral and cellular cytokines, the latter in part induced by KSHV, promote inflammatory infiltrates (vMIP, vIL-6), angiogenesis (vMIP, vIL-8R), and enhance spindle cell proliferation (vIL-6, vIL-8R via VEGF).

The reliable presence of KSHV in the spindle cells may point to additional factors beyond those induced through the 1%–3% productively infected cells, voting for a role more compatible with a typical "oncogenic transformation" model by latently expressed viral genes. Starting from the close relationship to EBV and herpesvirus saimiri, it is intriguing to assume that transmembrane proteins mimicking constitutively active receptors mechanisms, similar to those identified in EBV and herpesvirus saimiri, might be relevant for KSHV pathogenesis. At first sight, KSHV genes K1 and K15 might fall into this category. However, attempts to detect expression of these genes in latently infected tumor cells remain unsuccessful.

Thus, KS may result from a complex interplay of both viral and cellular cytokines and angiogenic factors, induced by a sustained inflammatory reaction initiated by up to 3% productively infected cells. Perhaps the viral cyclin and other latency-associated proteins such as LANA-1 might further enhance the proliferation of KS cells and favor the development of truly malignant cells by indirect means, e.g., the reduced control of accidental DNA damage. As KS is an unusual malignancy, resembling hyperplastic, angioproliferative inflammatory changes rather than true sarcoma, such a multistep/multifactorial model might be more compatible with KS pathogenesis than classical transformation models by viral oncogenes, as described for EBV, herpesvirus saimiri, and possibly for KSHV-associated B-cell malignancies (PEL).

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23

Gammaherpesviruses entry and early events during infection

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The two human gammaherpesviruses, Epstein–Barr virus (EBV), a gamma 1 lymphocryptovirus and Kaposi's sarcoma associated virus (KSHV), a gamma 2 rhadinovirus, have many features in common. They share an architecture that is typical of all members of the herpesvirus family, they share an ability to establish latency in lymphocytes, and they are both initiators or potentiators of human tumors. For the virologist some of the challenges they present are the same, in particular the relative dearth of fully permissive, easily manipulated cell culture systems for study. In this respect the many years of work on EBV provided an initial roadmap to accelerate study of KSHV. However, the strategies that the viruses use for cell infection and replication provide not only interesting reflections of common ancestry, but also interesting contrasts in adaptation to unique cellular niches in their human hosts.

Target cells for EBV

EBV can infect a variety of cell types under different circumstances, including T-cells, NK-cells, smooth muscle cells and possibly follicular dendritic cells (Rickinson and Kieff, 2001). However, B-lymphocytes and epithelial cells are its two major targets. B-cells are the primary reservoir of virus in persistently infected individuals and it is likely, although not certain, that the first cell infected in vivo is an epithelial cell. There has been some controversy over whether EBV normally infects epithelial cells during the courses of a primary infection or whether the virus infects epithelial cells only in the context of oncogenesis (nasopharyngeal carcinoma) or extreme immune dysfunction (oral hairy leukoplakia). Detection of EBV in epithelial tissue of healthy donors or patients with acute infectious mononucleosis has not always been consistent (Anagnostopoulos *et al.*, 1995: Frangou *et al.*, 2005; Lemon *et al.*, 1977; Niedobitek *et al.*, 1989; Pegtel *et al.*, 2004; Sixbey *et al.*, 1984; Venables *et al.*, 1989). However, the preponderance of findings and the characteristics of virus shed in saliva of healthy carriers support a role for epithelial cells in amplification of virus during primary and persistent infection (Jiang *et al.*, 2006). The clear association of the virus with significant epithelial pathology and its ability to infect epithelial cells in vitro either as free virus (Tugizov *et al.*, 2003; Turk *et al.*, 2006) or as B-cell associated virus (Imai *et al.*, 1998, Shannon-Rowe *et al.*, 2006) also reinforce the position that failures to detect virus may represent technical difficulties rather than reality.

B cells have been most extensively studied because the immortalizing effects of virus are so striking in this cell type, because B-cells are easier to isolate and because, after EBV infection, they are extremely easy to grow as latently infected cells. They retain episomes well in culture and are the best source of cell-free virus. Reactivation occurs spontaneously in a small subpopulation of some semipermissive B cell lines and can be induced in larger numbers of cells by agents such as phorbol esters, sodium butyrate or by cross-linking of immunoglobulin on the cell surface (Kieff and Rickinson, 2001). Epithelial cells typically lose episomes in vitro, but recent derivation of recombinant viruses that carry drug resistant markers has allowed selection of cells that can support episomal maintenance. The degree of lytic replication that occurs in epithelial cells varies from cell line to cell line. In many cell lines virus remains latent unless reactivated by treatment with inducing agents, but there is a recent report of extensive lytic replication in two polarized cell lines (Tugizov *et al.*, 2003).

Target cells for KSHV

The in vivo host cell range of KSHV is not yet fully characterized, but appears to be broad in that KSHV viral DNA and

transcripts have been detected in B-cells from the peripheral blood, B-cells in primary effusion lymphomas (PEL) or body-cavity based B-cell lymphomas (BCBL) and multicentric Castleman's disease (MCD), flat endothelial cells lining the vascular spaces of Kaposi's sarcoma (KS) lesions, typical KS spindle cells, CD45+/CD68+ monocytes in KS lesions, keratinocytes, and epithelial cells (Antman and Chang, 2000; Dourmishev *et al.*, 2003; Ganem, 1998; Sarid *et al.*, 1999; Schulz *et al.*, 2002). KSHV DNA is present in a latent form in the vascular endothelial and spindle cells of KS tissues and expresses the latency associated LANA1 (ORF 73), cyclin D (ORF72), vFLIP (ORF 71) and K12 genes. However, virus DNA is lost within a few passages of cells cultured from the KS lesions. This is reminiscent of the loss of EBV episomes from nasopharyngeal carcinoma cells in tissue culture and the reason for it is not currently known. KSHV DNA is found in the CD19+ peripheral blood B cells of KSHV seropositive individuals and the detection of both lytic and latent forms in B- cells of KS patients suggests that CD19+ B-cells may be a primary reservoir for persistent infection (Antman and Chang, 2000; Dourmishev *et al.*, 2003; Schulz *et al.*, 2002).

Cell lines with B-cell characteristics such as BC-1, HBL-6, JSC, BCBL-1 and BC-3 have been established from PEL tumors (Dourmishev *et al.*, 2003). The BC-1, HBL-6 and JSC cells carry KSHV and EBV genomes, while BCBL-1 and BC-3 cells carry only KSHV genome. KSHV exists in a latent state in these BCBL cells and expresses the latency associated ORF73, ORF72, K13, K12, K15 and ORF 10.5 genes. In parallel with EBV, spontaneous reactivation and expression of lytic cycle proteins occurs in 1% to 5% of BCBL cells. The lytic cycle can also be induced in about 20% to 30% of cells by phorbol esters, sodium butyrate and the lytic cycle switch protein known as RTA encoded by KSHV ORF 50 (Antman and Chang, 2000; Dourmishev *et al.*, 2003; Liao *et al.*, 2003; Schulz *et al.*, 2002)

KSHV also has a much broader in vitro tropism than EBV. The virus can infect human B, lymphocytes, endothelial, epithelial, and fibroblast cells, as well as a variety of animal cells such as owl monkey kidney cells, baby hamster kidney fibroblast cells, Chinese hamster ovary (CHO) cells, and mouse fibroblasts (Akula *et al.*, 2001b; Bechtel *et al.*, 2003; Blackbourn *et al.*, 2000; Ciufo *et al.*, 2001; Dezube *et al.*, 2002; Lagunoff *et al.*, 2002; Moses *et al.*, 1999; Naranatt *et al.*, 2003; Renne *et al.*, 1998; Vieira *et al.*, 2001). Unlike EBV, infection of primary B-cells by KSHV does not result in immortalization. However, like EBV in vitro infection by KSHV is characterized by the expression of latency associated genes and the absence of productive lytic replication. After activation with phorbol esters or ORF 50 protein, lytic replication is supported by many cells including primary human microvascular dermal endothelial cells (HMVEC-d), human umbilical vein endothelial cells (HUVEC), human foreskin fibroblast cells (HFF), human endothelial cells immortalized by telomerase (TIME) or the E6/E7 proteins of human papilloma virus, monkey kidney cells and mouse fibroblasts (Bechtel *et al.*, 2003).

Detection of KSHV latency associated nuclear antigen (LANA-1) encoded by ORF 73 after 2 days post-infection has led to the notion that the establishment of latency is the default pathway of infection (Bechtel *et al.*, 2003; Lagunoff *et al.*, 2002; Schulz *et al.*, 2002; Tomescu *et al.*, 2003). However, in vitro KSHV latent infection in primary fibroblasts, endothelial cells, or non-adherent B-cells is unstable, and viral DNA is not efficiently maintained (Tomescu *et al.*, 2003). Even during primary infection of endothelial and other cells, the proportion of KSHV-infected cells decreases over time (Grundhoff and Ganem, 2004). Loss of genomes begins approximately 4 days after infection and by 3 weeks fewer than 15% of the cells remain positive for LANA1 (Tomescu *et al.*, 2003). Whether the wild-type KSHV from the saliva of infected individuals or KSHV isolates from Africa have similar properties and tropisms remains unknown at the present time.

Virion structure

The virions of EBV and KSHV are at least superficially very similar to those of other herpesviruses. Each consists of a linear double stranded genome, an icosahedral capsid surrounded by an amorphous layer of tegument proteins and a lipid envelope carrying multiple unique glycoprotein species.

The KSHV genome is made up of a unique long region (LUR) of about 145kb flanked by varying numbers of terminal repeats. A large region of the KSHV genome is conserved among herpesviruses, and is colinear with both the gamma 1 EBV and the gamma 2 herpesvirus saimiri (HVS) (Neipel *et al.*, 1997; Russo *et al.*, 1996). It encodes more than 90 open reading frames (ORFs), designated ORFs 4 to 75 for their homology to those of HVS (Neipel *et al.* 1997; Russo *et al.*, 1996). Divergent regions in-between the conserved gene blocks contain more than 20 KSHV unique genes which are designated with the prefix K.

The EBV genome is slightly larger than that of KSHV at approximately 180 kb. However, it includes a large, internal, tandemly reiterated 300 kb repeat known as IR1, a number of smaller internal repeats (IR2-IR4) and 500 bp terminal repeats arranged in tandem, all of which can vary in number in different strains of viruses. The more than 90 ORFs of

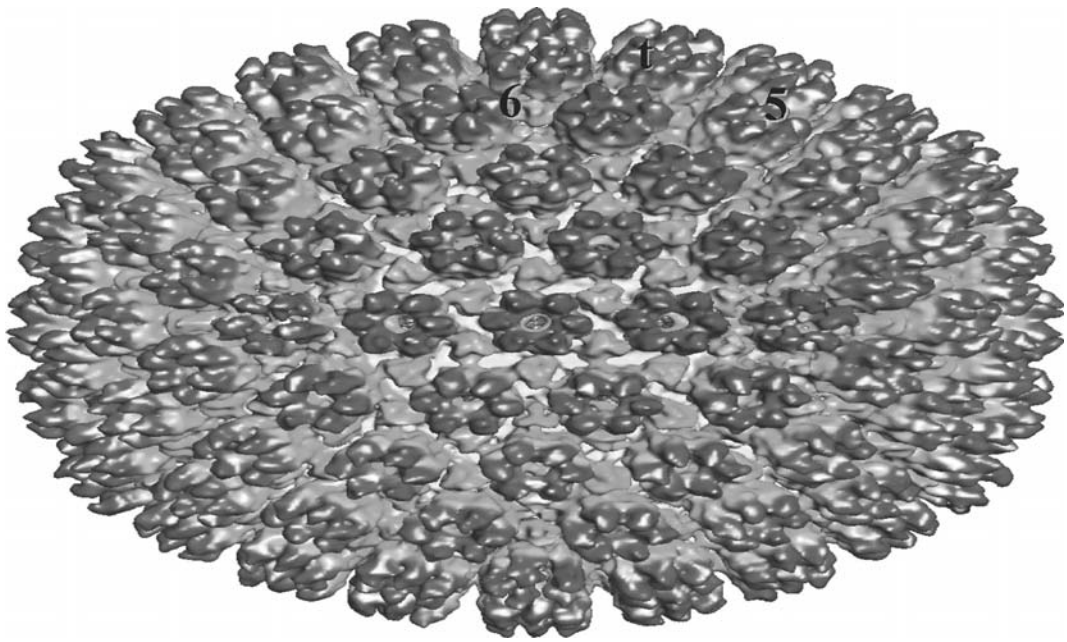

Fig. 23.1. 3D structure of the KSHV capsid at 24-Å resolution by electron cryomicroscopy. The capsid is shown as shaded surface color-coded according to particle radius. The three structural components of the capsid are indicated, including 12 pentons ("5"), 150 hexons ("6") and 320 triplexes ("t"). (Wu *et al.*, 2000 with permission). (See color plate section.)

EBV are named for the fragment within the original *Bam*H 1 restriction map of the B95–8 genome (Baer *et al.*, 1984) in which they originate and their direction of transcription. The BALF4 ORF, for example, is the fourth leftward reading ORF originating in the *Bam*H 1 A fragment.

Cryoelectron microscopy analyses reveal that KSHV capsid has the same $T = 16$ triangulation number, capsid architecture, structural organization and size as herpes simplex virus (HSV) and the human cytomegalovirus (HCMV) (Lo *et al.*, 2003; Trus *et al.*, 2001; Wu *et al.*, 2000) (Fig. 23.1). As in other herpesviruses, the capsids have a typical icosahedral shell and are composed of four structural proteins encoded by KSHV ORFs 25, 26, 62 and 65. The major capsid protein encoded by ORF 25 forms the hexameric and pentameric capsomers. The capsid floor between the hexons and pentons is formed by the heterotrimeric complexes composed of one molecule of the ORF62 protein and two molecules of the ORF26 protein. Both these proteins show significant amino acid sequence homology to the capsid proteins of alpha- and betaherpesviruses. The fourth protein, ORF65, is a small basic and highly antigenic protein, lacks significant sequence homology with its structural counterparts from the other subfamilies; however, similar to the small basic capsid protein VP26 of HSV, KSHV ORF 65 decorates the surface of the capsids. Lytic replication of KSHV leads to the formation of at least three capsid species, A, B, and C, and the A capsids are empty, B capsids contain an inner array of a fifth structural protein, ORF17.5, and C capsids contain the viral genome (Lo *et al.*, 2003; Trus *et al.*, 2001; Wu *et al.*, 2000).

No similar detailed analyses of the EBV capsid have yet been done, although transmission electron microscopy reveals a structural organization and size similar to other herpesviruses. Very little is known about the individual capsid proteins and assignments of ORFs as encoding capsid proteins have been made until recently only by homology with known HSV capsid proteins. Even less has been hypothesized about the composition of the tegument as few EBV ORFs have homology to any of the known HSV tegument proteins. The only EBV protein, which has been consistently described as a tegument protein is p140, which is thought to be encoded by the BNRF1 ORF (Hummel and Kieff, 1982). This ORF is described as having homology with *N*-formylglycinamide ribotide amidotransferases of *Eschericia coli* and *Drosophila melanogaster* (Russo *et al.*, 1996), but the significance of the homology is unknown. The first proteomic analysis of the proteins in the virion particle promises to stimulate new research in this understudied area (Johannsen *et al.*, 2004).

Virus structural proteins involved in entry

In contrast, considerable effort has been expended on analysis of the biochemistry and function of the envelope

Table 23.1. Genes encoding membrane proteins that are expressed only in the lytic cycle

EBV protein/gene	KSHV protein/gene	EBV function	KSHV function
gp350/220/BLLF1	—	attachment to B cell receptor CR2	—
gH/BXLF2	gH/ORF22	virus cell fusion; attachment to epithelial cell receptor/coreceptor; complexes with gL	virus entry; complexes with gL
gL/BKRF2	gL/ORF47	chaperone for gH	chaperone for gH
gp42/BZLF2	—	interaction with B cell coreceptor HLA class II	—
gB/BALF4	gB/ORF8	assembly/exit/fusion	attachment to cells via heparan sulfate; interacts with integrins; virus entry; egress
gN/BLRF1	gN/ORF53	codependent on gM for processing; complex of gNgM required for production of enveloped virions	complexes with gM
gM/BBRF3	gM/ORF39	complexes with gN; complex required for production of enveloped virions	codependent on gN for processing; complex inhibits cell fusion
BMRF2	—	interacts with integrins; important to infection of polarized epithelial cell lines	—
gp150/BDLF3	—	unknown	—
p38/BFRF1		homologue of alphaherpesvirus UL34 proteins	
	?ORF67	interacts with BFLF2 protein (UL31 homologue) localizes to the nuclear rim, facilitates nuclear egress	?
gp78/BILF2	—	unknown	—
BILF1	—	constitutively active G protein-coupled receptor	—
—	gpK8.1A/K8.1	—	attachment to cells via heparan sulfate; virus entry
—	gpK8.1B/K8.1	—	unknown
—	OX-2/K14	—	adhesion of infected cells
—	K1/K1	—	signal induction
—	K15/K15	—	signal induction

proteins. These are the proteins that are critically needed to mediate attachment, entry, assembly and egress of virus and the majority of them are glycoproteins. The different tropisms of herpesviruses are potentially due to unique complements of these glycoproteins as well as subtle differences among those that are conserved.

The precise number of EBV membrane proteins that are exclusively expressed during the lytic cycle is not yet known, but there is evidence for at least twelve (Table 23.1). Five of these are glycoproteins that have homologues in all herpesvirus studied to date and are now commonly referred to by the naming system originally developed for the prototype alphaherpesvirus, herpes simplex virus. They are gB gH, gL, gM, and gN (Hutt-Fletcher, 2002). The remaining seven proteins are named for their apparent mass or referred to by the name of the open reading frame (ORF) that encodes them. One, p38, the product of the BFRF1 gene (Farina *et al.*, 2000) is also conserved among all the herpesvirus families and is the homolog of the most widely studied alphaherpesvirus UL34. It is not glycosylated and is not found in the virion particle (Farina, 2004; Johannsen *et al.*, 2004). It interacts with the BFLF2 gene product, the homolog of alphaherpesvirus UL31 (Gonella *et al.*, 2005; Lake and Hutt-Fletcher, 2004) and like its homologs in other herpesviruses its major role is probably to facilitate exit of the newly formed EBV nucleocapsid from the nucleus (Farina *et al.*, 2005). The remaining six are glycoproteins that are unique to the gammaherpesviruses. Glycoproteins gp150 (Kurilla *et al.*, 1995; Nolan and Morgan, 1995), gp78 (Mackett *et al.*, 1990) and the product of the BILF1 gene, which is glycoprotein with an apparent mass of approximately 50 kDa (Paulson *et al.*, 2005), have counterparts only in lymphocryptoviruses (Rivailler *et al.*, 2002). Glycoproteins gp350/220 (Hummel *et al.*, 1984), gp42 (Li *et al.*, 1995) and the BMRF2 protein (Tugizov *et al.*, 2003) have counterparts in lymphocryptoviruses (Rivailler *et al.*, 2002) and in certain of the rhadinoviruses (Russo *et al.*, 1996; Telford *et al.*, 1995; Virgin *et al.*, 1997). Glycoproteins gp350/220, gp42 and the BMRF2 protein, together with the conserved glycoproteins gB, gH and gL, are all involved in virus entry

as described below. It also is possible that complexes of gN and gM may play a role in this process although their major role is probably in envelopment and egress of newly made virus as described in Chapter 25 (Lake and Hutt-Fletcher, 2000). The phenotype of a virus that lacks gp150 is little changed from wild type virus, except that its ability to infect epithelial cells is very slightly enhanced (Borza and Hutt-Fletcher, 1998). The BILF1 gene product functions as a constitutively active G protein-coupled receptor. It is not found in the virion (Johannsen *et al.*, 2004), but may play a role in modulating intracellular signaling pathways (Beisser *et al.*, 2005). Nothing is known about possible functions of gp78.

KSHV also expresses the five conserved herpesvirus glycoproteins. (Table 23.1). Open reading frames 8, 22, 47, 39 and 53 encode glycoproteins gB, gH, gL, gM and gN, respectively (Akula *et al.*, 2001a; Baghian *et al.*, 2000; Naranatt *et al.*, 2002; Neipel *et al.*, 1997; Russo *et al.*, 1996). HHV-8 also encodes for additional glycoproteins such as gpK8.1A, gpK8.1B, K1, K14 and K15 that are expressed during lytic replication (Birkmann *et al.*, 2001; Chandran *et al.*, 1998; Neipel *et al.*, 1997; Russo *et al.*, 1996). Studies have shown that gB, gH/ gL, gM/gN and gpK8.1A are virion-envelope associated glycoproteins (Akula *et al.*, 2001a; Baghian *et al.*, 2000; Koyano *et al.*, 2003; Naranatt *et al.*, 2002; Neipel *et al.*, 1997; Russo *et al.*, 1996; Zhu *et al.*, 1999).

Virus attachment

Entry of enveloped viruses into cells involves at least two events, attachment to the cell surface and penetration through the cell membrane. Herpesviruses, not unexpectedly given the large number of different proteins found in a herpesvirus envelope, use multiple different proteins to complete the entry process. Increasingly, in recent years it has also become apparent not only that multiple proteins are involved in entry, but also that the complement of proteins that is used can vary considerably when different cells are the target of infection and different molecules are available for interaction with the virus.

Attachment of EBV

Attachment of EBV to B-cells has been known for many years to be mediated by a high affinity (Moore *et al.*, 1989) protein–protein interaction between the virus glycoprotein gp350/220 and the complement receptor type 2, CR2 or CD21 (Fingeroth *et al.*, 1984; Frade *et al.*, 1985; Nemerow *et al.*, 1985). Glycoprotein gp350/220 is an abundant, highly glycosylated type 1 membrane protein and has a dual nomenclature because its gene is expressed in two alternatively spliced forms with masses of approximately 350 and 220 kDa (Beisel *et al.*, 1985; Hummel *et al.*, 1984). The splice maintains the reading frame of the protein and results in the loss of residues 500 to 757 of the full-length 907 amino acid form which contain three repeats of a 21 amino acid motif with amphipathic characteristics. The splice does, however, retain the CR2 binding domain at the amino terminus of the molecule. Although not definitively mapped, this binding site is thought to include a short sequence of 21 amino acids that is very similar to the proposed binding sequence of the natural ligand of CR2, the C3d component of complement (Lambris *et al.*, 1985; Nemerow *et al.*, 1989; Tanner *et al.*, 1988). The functional significance of the existence of the two spliced forms of the EBV protein, if any, is unclear. However, the initial interaction between gp350/220 and CR2 tethers the virus approximately 50 nm from the B -cell surface (Nemerow and Cooper, 1984) so one possibility is that exchange of the larger for the smaller form might bring the virus a little closer to the cell membrane.

CR2 is a type 1 membrane protein and a member of a large family of proteins involved in tissue repair, inflammation and the immune response that is characterized by structural modules known as short consensus repeats (SCR). The EBV binding site has been very precisely mapped to the amino terminal SCR-1 and SCR-2 (Martin *et al.*, 1991). The tandem repeats, which comprise the entire extracellular domain of CR2, are 60–75 amino acids in length each forming discrete structural units (Moore *et al.*, 1989) that probably provide some segmental flexibility to the molecule (Weisman *et al.*, 1990) and that may also be important to positioning the virus for entry.

The ligand/receptor pair that is responsible for attachment of EBV to an epithelial cell is much less clear. Early models proposed that epithelial cells were infected when B-cells fused with epithelial membranes, but there are no data that either refute or confirm this possibility (Bayliss and Wolf, 1980). Some epithelial cell lines express at least low levels of CR2 (Fingeroth *et al.*, 1999; Imai *et al.*, 1998) and stable expression from a cDNA clone of CR2 renders a significant proportion of the cells permissive to infection (Li *et al.*, 1992). However, the physiological relevance of this is uncertain. Identification of CR2-expressing epithelial cells in vivo has been confounded by the fact that the monoclonal antibodies used to make the determinations cross-react with an unrelated epithelial cell protein (Young *et al.*, 1989). Cells that carry the polymorphic IgA receptor can be infected with virus that is coated with IgA specific for gp350/220 (Sixbey and Yao, 1992). This may be particularly relevant to infection of cells at the basolateral surface in an immune host, although in polarized cells virus was transported intact from the basolateral to the apical surface

so it may be more important for virus shedding than virus infection (Gan *et al.*, 1997).

Epithelial lines also express an as yet unidentified molecule that facilitates virus binding via a complex of gH and gL (Molesworth *et al.*, 2000; Oda *et al.*, 2000). Virus lacking gHgL loses the ability to bind to these lines and soluble forms of gHgL can be shown to attach specifically (Borza *et al.*, 2004). However, infection rates are low and it is possible that this interaction represents inefficient use, in the absence of a primary attachment receptor, of a coreceptor that is more important for penetration (see below). Most recently, an interaction between the BMRF2 protein and $\alpha 5\beta 1$ integrins on the basolateral surfaces of polarized epithelial cells has been reported to lead to high levels of infection and lytic replication in vitro (Tugizov *et al.*, 2003). This observation is particularly compelling since polarized epithelial cells are probably closer to the environment encountered by virus in vivo. The BMRF2 protein is predicted to span the membrane many times and one of the predicted extracellular loops contains an RGD motif. Further work will be necessary to determine whether use of this motif is most relevant to attachment or penetration of virus, but it may provide parallel to the use of an RGD sequence in KSHV gB (see below) for entry. Unlike KSHV and many other herpesviruses, discussed in the next section, EBV is not known to encode any heparan sulfate binding proteins.

Attachment of KSHV

The broad cellular tropism of KSHV which binds to a variety of target cells such as human B, endothelial and epithelial cells, and monocytes (but not T and NK cells), as well as a variety of animal cells (Akula *et al.*, 2001b; Dezube *et al.*, 2002) may be in part due to its interaction with the ubiquitous cell surface heparan sulfate (HS) (Akula *et al.*, 2001b). The initial virus–cell interactions of many other herpesviruses including herpes simplex types 1 and 2, pseudorabies virus, bovine herpesvirus 1, human cytomegalovirus, human herpesvirus 7 and bovine herpesvirus 4 also involve binding to HS. The first indication that HS might be involved in KSHV infection of target cells came from the serendipitous observation that infection of primary HMVEC cells was difficult in the presence of the heparin that is used in the growth medium of these cells. Further analyses showed that KSHV infection can be inhibited in a dose-dependent manner by soluble heparin, a glycosaminoglycan closely related to HS, but not by chondroitin sulfates A and C (Akula *et al.*, 2001b). Infectivity is reduced by enzymatic removal of cell surface HS with heparinase I and III, virus binding is blocked or displaced by soluble heparin and binding is drastically reduced on CHO cells that are deficient in HS (Akula *et al.*, 2001b). The interaction with HS may be the first set of ligand-receptor interactions that concentrates KSHV on the cell surface where it can subsequently bind to one or more additional host cell molecules that are essential to the entry process.

Two consensus motifs for the heparin binding domain (HBD) of polypeptides have been proposed, XBBXBX and XBBBXXBX, where B is a positively charged basic amino acid (lysine, arginine or histidine) flanked by an additional positively charged residue separated by hydrophobic amino acids "X" (Akula *et al.*, 2001a). Heparin-binding proteins often contain more than one of these sequences, and analysis of HBD of several proteins suggest that the negatively charged sulfate or carboxylate groups on heparin may interact via electrostatic interactions with positively charged cationic residues in a protein or peptide (Akula *et al.*, 2001a). Predictive analysis of KSHV sequences revealed putative HBD in the extracellular domains of HHV-8 gB and gpK8.1A. KSHV-gB contains the BXXBXBBXBB (^{108}HIFKVRRYRK^{117}) type HBD, which is conserved throughout the $\gamma 2$ herpesviruses (Akula, 2001a), and gpK8.1A possess two possible, although atypical heparin-binding motifs, gpK8.1A-H1 (^{150}SRTTRIRV^{157}, XBXXBXBX) and gpK8.1A-H2 (^{182}TRGRDAHY^{189}, XBXBXXBX) (Wang *et al.*, 2001).

The KSHV K8.1 gene is positionally colinear to glycoprotein genes in other members of the gammaherpesvirus subfamily including the EBV gene encoding gp350/gp220, the gp150 gene of murine gamma herpesvirus 68, the ORF 51 gene of HVS, and the BORFD1 gene of bovine herpesvirus 4 (Neipel *et al.*, 1997; Russo *et al.*, 1996). The K8.1 gene (genomic nucleotide position 76 214 bp – 76 808 bp) encodes a 197-aa long ORF with a predicted molecular weight of about 22 kDa, with a N-terminal signal sequence and five putative *N*-glycosylation sites, but without any transmembrane sequence. Screening of a cDNA library from TPA induced BCBL-1 library with a HIV+KS+ serum identified two cDNAs encoded by the gpK8.1 gene (Chandran *et al.*, 1998). Analyses of these cDNAs show that the gpK8.1 gene encodes two ORFs, designated gpK8.1A and gpK8.1B, from spliced messages. The larger cDNA is 752 bp long (76 214 – 76 941 bp) and utilizes the polyadenylation signal sequence (AATAAA) at position 77 013 bp. The 228-aa long encoded protein is designated gpK8.1.A which contains a signal sequence, transmembrane domain, and four *N*-glycosylation sites.

The first 142 amino acids encoded by the gpK8.1A cDNA are identical to the genomic gpK8.1 ORF sequence. This cDNA is derived from a transcript with a 95bp sequence spliced out [CAG/(GT)GTAT donor site and TCTAC(AG)/G

acceptor site] and ends at the genomic nucleotide position 76 941 bp, which is 187bp beyond the end of genomic gpK8.1 ORF. This has resulted in the generation of a transmembrane domain not seen in the genomic gpK8.1 ORF. The smaller 569 bp long cDNA encodes the gpK8.1B, with a 183-bp sequence spliced out. The splice acceptor site for the ORF gpK8.1B transcript is the same as the gpK8.1A ORF; however, the splice donor site [CGA/(GT)GAGT] for the gpK8.1B cDNA is upstream of the splice donor site of the gpK8.1.A cDNA resulting in frame deletion of 61 amino acids in the smaller ORF. The resulting 167 aa long ORF is a typical class I glycoprotein with a cleavable signal sequence, a transmembrane domain, three putative *N*-glycosylation sites and is predicted to code for a protein of about 18.5 kDa. Except for an amino acid change near the splice site (S to R), the gpK8.1B shares identical amino acid sequences with the gpK8.1.A. Both gpK8.1A and gpK8.1B contain N- and O-linked sugars, and gpK8.1A is the predominant form detected within the infected cells and the virion envelopes (Chandran *et al.*, 1998; Neipel *et al.*, 1997; Zhu *et al.*, 1999). Both are immunogenic proteins (Zhu *et al.*, 1999).

The 845 amino acid KSHV-gB ORF includes a predicted signal sequence between residues 1–23, a predicted transmembrane domain between amino acids 710–729 and 13 potential *N*-glycosylation sites. There is a potential proteolytic cleavage site (RKRR/S) at amino acid position 440–441, and cleavage at this site would result in two proteins with predicted masses of about 48 and 45 kDa (Akula *et al.*, 2001a; Baghian *et al.*, 2000). Experimentally the protein has been shown to be expressed on the surface of the infected cell and in virion envelopes (Akula *et al.*, 2001a; Baghian *et al.*, 2000). It is synthesized as a 110 kDa precursor protein, undergoes cleavage and processing, and the envelope-associated form consists of 75 and 54 kDa polypeptides that form disulfide-linked heterodimers and multimers (Akula *et al.*, 2001a).

Several lines of evidence indicate that KSHV-gB and gpK8.1A bind to cell surface HS molecules (Akula *et al.*, 2001a; Birkmann *et al.*, 2001; Wang *et al.*, 2001). Binding of soluble forms of the proteins made in baculovirus is saturable and can be blocked by soluble heparin (Wang *et al.*, 2001, 2003). Full-length gB and gpK8.1A in the virion envelope specifically bind heparin-agarose, and can be eluted by high concentrations of soluble heparin, but not by chondroitin sulfates (Akula *et al.*, 2001a; Wang *et al.*, 2001). KSHV-gpK8.1A binds to heparin with an affinity comparable to that of glycoproteins B and C of herpes simplex virus (Birkmann *et al.*, 2001) and the gpK1A binds more strongly than gB (Wang *et al.*, 2003). Even though the involvement of KSHV-gB residues 108–117 and gpK8.1A residues 150–157 in binding to HS-like moieties has been convincingly demonstrated, it is also possible that other weak and/or high affinity HBDs may appear in HHV-8 gB and gpK8.1A in their native quaternary structures if basic amino acids separated linearly are juxtaposed, forming a typical HBD. The presence of two or more heparin-binding glycoproteins within a single virus is not unprecedented, since the well-studied human α-and β-herpesviruses contain at least two HS binding glycoproteins e.g. gB and gC for herpes simplex 1 and 2, gB and gCII for human cytomegalovirus, and gB and gp65 for human herpesvirus 7. The presence of two-HS binding proteins in KSHV re-emphasizes the importance of cell surface HS for attachment of many, although not all, herpesviruses.

Penetration

Penetration of any enveloped virus into a cell involves fusion of the virion envelope with the membrane of the cell and can occur either at the cell surface or after endocytosis. Endocytosis affords a convenient and often rapid system of transit across the plasma membrane and through the cytoplasm for delivery of viral cargo to the vicinity of the nucleus (Sieczkarski and Whittaker, 2002; Whittaker, 2003). The best understood paradigm for virus cell fusion is provided by the RNA viruses such as the human immunodeficiency virus, which fuses at the cell surface, and influenza virus, which fuses with the endocytic vesicle. The virus glycoproteins that mediate fusion are made as single type 1 membrane proteins, but are cleaved during processing to create two species which reassociate in a metastable state (Colman and Lawrence, 2003). The fragment that retains the transmembrane domain includes a hydrophobic sequence or "fusion peptide" that can be triggered by conformational changes to insert into an opposing cell membrane and initiate formation of a fusion pore. The conformational change in the human immunodeficiency virus is triggered by interaction with coreceptors, the conformational change in the influenza virus fusion protein is triggered by exposure to the low pH of the endosome. However, no clear-cut paradigm has yet been identified for any herpesvirus. Fusion appears to require cooperation between several unique protein species, none of which include readily identifiable "fusion peptides" and the site at which fusion occurs varies from virus to virus and even cell type to cell type.

Penetration by EBV

Penetration of EBV into B cells and epithelial cells is significantly different both in terms of the virus and cell proteins

involved and in terms of the routes that are used. Attachment of EBV to the B cell surface via CR2 stimulates endocytosis of virus into thin-walled non-clathrin coated vesicles (Nemerow and Cooper, 1984; Tanner *et al.*, 1987) and fusion occurs in a low pH environment. Exposure to low pH is not an essential requirement, but endocytosis does appear to be necessary as virus fails to fuse with B cells treated with the endocytosis inhibitors chlorpromazine and sodium azide. In contrast, fusion with an epithelial cell occurs at neutral pH and is resistant to treatment with either chlorpromazine or sodium azide (Miller and Hutt-Fletcher, 1992).

Fusion of the EBV envelope with the B cell requires at least three and probably four virus glycoproteins, gB, gH, gL and gp42. EBV gB is a 857-residue protein that shares some structural, although little sequence homology with its counterparts in other herpes viruses (Gong *et al.*, 1987). The positions of many of the cysteine residues in gB are conserved. Like gB of HHV-8 it undergoes cleavage to produce two polypeptides of approximately 56 and 80 kDa that are linked by disulfide bonds (Johannsen *et al.*, 2004; C. M. Lake and L. M. Hutt-Fletcher, unpublished data). Although some strains of EBV carry very little gB in the virion (Gong and Kieff, 1990) and, as discussed in Chapter 25, EBV gB plays a very important role in virus assembly (Lee and Longnecker, 1997), recent work in which different combinations of virus proteins were expressed and examined for their abilities to fuse cell membranes indicates that EBV gB is also an essential part of the fusion machinery (Haan *et al.*, 2001).

The remaining three proteins that are known to be required for fusion with B cells, gH, gL and gp42, form a non-covalently linked complex in virus (Li *et al.*, 1995). Liposomes that contain all virus envelope proteins, except gH, gL and gp42, bind to receptor positive cells but fail to fuse (Haddad and Hutt-Fletcher, 1989) and recombinant virus that lacks all three can bind to but cannot penetrate B cells (Molesworth *et al.*, 2000). Glycoprotein gH, the largest of the three is a 708 residue type 1 membrane protein with five potential *N*-linked glycosylation sites and it carries about 10 kDa of *N*-linked sugar (Baer *et al.*, 1984; Heineman *et al.*, 1988; Oba and Hutt-Fletcher, 1988). Although members of the gH family of proteins share little sequence homology, if aligned at a conserved *N*-linked glycosylation site at the carboxyterminus they show a co-linearity of cysteine residues that suggests a conservation of secondary structure (Klupp and Mettenleiter, 1991). Each member is also dependent on the smaller membrane protein, gL, for folding and transport through the cell. The EBV gL is a 137-residue glycoprotein of approximately 25 kDa that remains anchored in the envelope by an uncleaved signal sequence (Li *et al.*, 1995; Yaswen *et al.*, 1993). Because of the dependence of gH on gL and because EBV in which expression of the gH gene is interrupted phenotypically lacks gL as well (Molesworth *et al.*, 2000), there are only few instances in which the functions of the two proteins can be separated.

Glycoprotein gp42, the third member of the complex required for penetration of a B cell, is not dependent on gHgL for processing and does not have counterparts in most herpesviruses (Li *et al.*, 1995). The gHgL complex in both human cytomegalovirus and human herpes virus 6 also include a third component known, respectively, as gO and gQ (Huber and Compton, 1998; Mori *et al.*, 2003), but these latter proteins, have no obvious homology to gp42. Only in the gammaherpesviruses are similar proteins predicted (Rivailler *et al.*, 2002; Telford *et al.*, 1995). EBV gp42 is a 223 residue type 2 membrane glycoprotein that has weak similarity to a C-type lectin (Spriggs *et al.*, 1996). The predicted signal sequence and transmembrane anchor lie between residues 7 and 28, the region of the molecule that is responsible for the interaction with gHgL lies between residues 40 and 58 (Wang *et al.*, 1998; Ressing *et al.*, 2005) and the carboxyterminal domain of the protein interacts with the variable region of the β chain of HLA class II (Spriggs *et al.*, 1996).

Several lines of evidence indicate that the interaction between gp42 and HLA class II is essential to B-cell infection. A monoclonal antibody to gp42 that blocks the interaction with HLA class II inhibits virus cell fusion (Miller and Hutt-Fletcher, 1988) and a monoclonal antibody to HLA class II that blocks gp42 binding neutralizes virus infection (Li *et al.*, 1997). A soluble form of gp42 in which the transmembrane domain is replaced with the Fc domain of human immunoglobulin competes with gp42 in virus for binding to HLA class II and blocks infection and B-cells that lack HLA class II cannot be infected unless HLA class II expression is restored (Li *et al.*, 1997). Finally, a recombinant virus that lacks gp42 fails to infect B-cells unless cells and bound virus are fused with polyethylene glycol (Wang and Hutt-Fletcher, 1998), or a soluble form of gp42 which lacks a transmembrane domain but which retains the ability to bind to gH and gL is added in *trans* (Wang *et al.*, 1998). Binding shows some allelic specificity (Haan and Longnecker, 2000) and the crystal structure of HLA class II bound to gp42 reveals that gp42 binds peripherally to the variable domain of the β-chain (Mullen *et al.*, 2002). A current minimalist model of B cell penetration would then suggest that, following attachment of virus via gp350 and CR2, gp42 interacts with HLA class II and that this event leads to triggering of the fusion machinery, gHgL and gB.

Fusion of virus with epithelial cells requires only gB and gHgL (McShane and Longnecker, 2004) and residues required for fusion can be distinguished from those

required for fusion with B cells (Omerovic *et al.*, 2005; Wu *et al.*, 2005). However, epithelial cells do not express HLA class II constitutively, and recombinant virus that lacks gp42 infects epithelial cells as well as wild-type virus. Not only is gp42 dispensable for infection of epithelial cells, its presence is also inhibitory. Stoichiometric analysis of wild-type virus demonstrates the presence of much larger amounts of gHgL than gp42 in the virion, implying that some complexes naturally lack or are low in gp42. Saturation of the complexes by addition of soluble gp42 in *trans* blocks epithelial infection. In addition infection of epithelial cells, but not B cells, can be blocked by antibodies that interact with gHgL or gH alone (Wang *et al.*, 1998). These findings have been interpreted to mean that there is a coreceptor on epithelial cells that can substitute for HLA class II and with which gHgL interacts in the absence of gp42. Soluble forms of gHgL bind saturably to epithelial cells. Scatchard analysis indicates the presence of as many as 200 000 high affinity receptors per cell with a K_D of approximately 5×10^{-9} M (L. Chesnokova, A. Morgan and L. Hutt-Fletcher, unpublished data). Whether or not this receptor is the same as that which can be used to attach virus to epithelial cells is not yet known. However, a minimal model of penetration of an epithelial cell suggests that following attachment an interaction of gHgL alone with a coreceptor triggers the activity of the fusion machinery.

The observation that gp42 is essential for B-cell infection but dispensable for epithelial infection suggested that changes in the stoichiometry of the gHgLgp42 complex would influence tropism of EBV for the two cell types and comparisons of virus made in HLA class II positive B-cells and HLA class II negative epithelial cells support the hypothesis that such changes might occur in vivo (Borza and Hutt-Fletcher, 2002). Virus made in HLA class II-negative epithelial cells can be as much as two logs more infectious for B-lymphocytes than the same amount of virus produced by an HLA class II-positive B-cell. Virus originating from either cell type binds equally well to CR2 on the B-cell surface, but virus made in the B-cell enters less efficiently. This appears to reflect the fact that in a class II-positive virus-producing cell some complexes containing gp42 interact with class II during biosynthesis and are targeted to the class II trafficking pathway where they are vulnerable to degradation. The resulting loss of three-part complexes from virus reduces the efficiency of class II-dependent entry. Such a loss does not occur in a class II-negative epithelial cell where virus has a relative increase in gp42 and an increased efficiency for class II-dependent entry and induction of HLA class II expression can reverse the phenotype. The levels of gp42 in virus also impact infection of epithelial cells via the class II-independent pathway. B cell virus is on average fivefold better at infecting epithelial cells than epithelial virus. These findings suggest that gp42 may function as a switch of virus tropism that might be relevant to spread of virus between tissues in vivo (Borza and Hutt-Fletcher, 2002) (Fig. 23.2). However, since the effects on B-cell infection are by far the most striking,

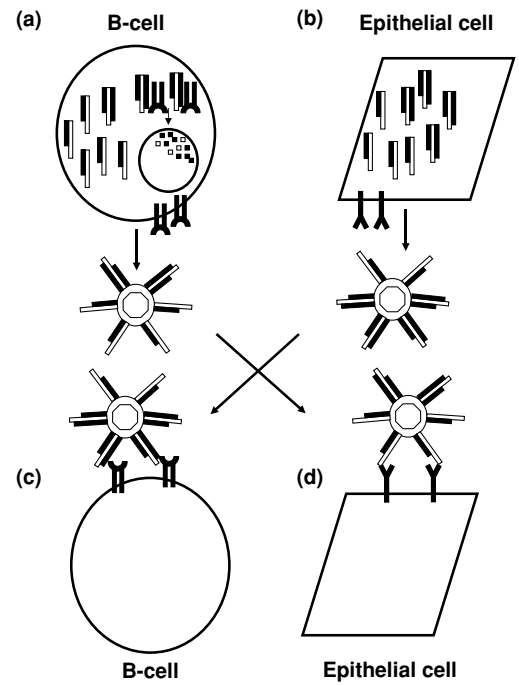

Fig. 23.2. Glycoprotein gp42 can function as a switch of EBV tropism. (a) EBV makes both three part gHgLgp42 complexes and two part gHgL complexes. When virus is made in a B-cell, some of the three-part gHgLgp42 complexes bind to HLA class II within the cell and as a result traffic along the same exocytic pathway as that followed by HLA class II. This includes passage through a vesicle rich in proteolytic enzymes which normally digests exogenous protein for presentation by HLA class II. The gHgLgp42 complexes bound to HLA class II are proteolytically digested in this compartment and as a result fewer of the three-part complexes are available for incorporation into virus. (b) In an epithelial cell which does not express HLA class II a larger number of three part complexes are available for incorporation into virus. (c) Entry into a B-cell requires an interaction between gp42 and HLA class II. The relative increase of gp42 in virus derived from an epithelial cell thus increases the ability of virus to infect a B-cell. (d) Infection of an epithelial cell requires an interaction with a gHgL coreceptor. Interaction of the gHgL complex with this coreceptor is blocked by gp42. The relative reduction of gp42 in virus derived from an HLA class II positive B-cell thus increases the ability of this virus to infect an epithelial cell. gHgL; gHgLgp42; HLA class II; gHgLR.

the biological effects may primarily be to drive into the B-cell pool any virus that initiates infection by replicating in an epithelial cell.

The events that occur concurrent with, and following, fusion of the virus and cell membrane that are necessary to facilitate transport, uncoating and delivery of the genome to the nucleus are largely unknown and currently can only be guessed at, based on what little is understood for other herpesviruses. There are, however, hints that additional envelope proteins may be involved in efficient delivery of infectious virus. Loss of the complex of gM and gN not only severely compromises envelopment and egress of EBV, as described in Chapter 25, but also leads to a defect in infection that cannot be rescued by treating bound virus with exogenous mediators of fusion. One possible explanation for this finding is that there is a defect in dissociation of envelope and tegument necessary for movement of virus toward the nucleus (Lake and Hutt-Fletcher, 2000).

Penetration by KSHV

KSHV enters the B-cell line BJAB (Akula *et al.*, 2001a), HFF (Akula *et al.*, 2003), the human epithelial line 293 (Inoue *et al.*, 2003; Liao *et al.*, 2003) and endothelial cells (Akula *et al.*, 2002) by endocytosis. HHV-8 virions can be visualized in endocytic vesicles of BJAB (Akula *et al.*, 2001b, 2003) within 5 min of infection where they fuse with the vesicle wall (Akula *et al.*, 2003), and viral capsids are found in the vicinity of nuclear membranes by 15 minutes after infection. Anti-KSHV- gB antibodies colocalize with virus-containing endocytic vesicles. In HFF cells, KSHV infection is significantly inhibited by the preincubation of cells with chlorpromazine HCl, which blocks endocytosis via clathrin-coated pits, but not by nystatin and cholera toxin B, which block endocytosis via caveolae and induce the dissociation of lipid rafts, respectively. Infection is also inhibited by blocking the acidification of endosomes by NH_4 Cl and bafilomycin A in HFF and 293 cells (Akula *et al.*, 2003; Liao *et al.*, 2003). These findings suggest that penetration of KSHV occurs in and requires a low pH intracellular environment. Further work is required to determine if virus takes the same route into other cell types.

The KSHV gHgL complex consists of a 120 kDa protein (gH) and a 41–42 kDa protein (gL) linked by non-covalent bonds and found both on the surface of the cell and in virions (Naranatt *et al.*, 2002). As in other herpesviruses, KSHV-gL is required for gH processing and intracellular transport and the complex is required for entry. Anti-gH and anti-gL antibodies neutralize KSHV infectivity, individually and more efficiently together, without having any effect on virus binding to target cells (Naranatt *et al.*, 2002). Deletion of 58 amino acids in the cytoplasmic tail of KSHV-gB removed the putative endocytosis signals (YXXΦ). Expression of this truncated KSHV-gB (gBMUT), but not the full length gB, can be detected on the surface of CHO cells (Pertel, 2002). Co-expression of KSHV- gBMUT, gH, and gL resulted in the fusion of CHO cells with 293 cells (Pertel, 2002). Further work is necessary to determine the role of KSHV gB, gH and gL mediated fusion in entry of target cells, but at this point it seems likely that the minimal fusion machinery of both KSHV and EBV comprises gB, gH and gL.

Much more is known about the involvement of KSHV gpK8.1 and gB in post binding events. Although these proteins are involved in the interaction with the cell surface HS molecules, even high concentrations of rabbit polyclonal and monoclonal anti-gB and anti-gpK8.1A antibodies which neutralize infection do not block the binding of KSHV to target cells (Akula *et al.*, 2001a; Zhu *et al.*, 1999). This implies a role for the proteins after attachment has occurred; possibly as a result of interaction with additional cell surface molecules. Among all the gB homologues sequenced to date, only KSHV-gB possesses an integrin-binding RGD motif at amino acids 27 to 29 which is predicted to be immediately adjacent to the putative signal sequence of the protein (Akula *et al.*, 2002). The RGD motif is the minimal peptide region of many extracellular matrix (ECM) proteins known to interact with subsets of host cell surface integrins. KSHV infectivity of fibroblasts and endothelial cells is neutralized by RGD peptides, by antibodies to α3 and β1 integrins, and by soluble α3β1 integrin (Akula *et al.*, 2002) and anti-gB antibodies immunoprecipitate a complex of virus and α3β1. At the same time, RGD peptides, anti-integrin antibodies and soluble integrins fail to block virus binding to adherent target cells such as human endothelial and fibroblast cells suggesting that KSHV uses the a3β1 integrin as one of the cell receptors or coreceptors for entry (Akula *et al.*, 2002). Expression of human α3 integrin also increases the infectivity of virus for CHO cells (Akula *et al.*, 2002).

Additional studies suggest that infection of fibroblasts or endothelial cells can also be neutralized by soluble αVβ3 and αVβ5 integrins with higher levels of neutralization with soluble α3β1 integrin. Virus binding and viral DNA internalization studies suggest that αVβ3 and αVβ5 integrins also play roles in KSHV entry and may expand the in vivo target cells for KSHV. Using an RTA-dependent reporter 293-T cell line (Inoue *et al.*, 2003) reported the inability of soluble α3β1 integrin and RGD peptides to block the infectivity of KSHV. However,

in this study virus was centrifuged with cell in the presence of polybrene which may account for the apparent discrepancy. Polybrene is a positively charged cation which can complex with the virus envelope and may bypass the need for a receptor. This property of polybrene is the basis for its use to increase the infectivity of many viruses and to deliver nucleic acid for gene therapy. The nature of other receptor(s) recognized by KSHV and the glycoproteins involved need to be evaluated further.

KSHV also utilizes the dendtric cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN; CD209) as a receptor for infection of myeloid DCs and macrophages (Rappocciolo *et al.*, 2006). DC-SIGN was required for virus attachment to these cells and DC-SIGN-expressing cell lines. KSHV binding and infection were blocked by anti-DC-SIGN monoclonal antibody and soluble DC-SIGN, and mannan, a natural ligand for DC-SIGN. The residual level of KSHV binding and infection in cells pretreated with anti-DC-SIGN antibodies in these studies were attributed to additional receptors for KSHV on these cells. Expression of DC-SIGN on B-lymphoblastoid cell lines (LCL) and K562 cells which are normally resistant to KSHV rendered them susceptible to KSHV infection (Rappocciolo *et al.*, 2006). Since neither of these cells expressed $\alpha 3\beta 1$ on their surface, this suggested that other molecules such as DC-SIGN may be involved in infection of these target cells.

Another recent study showed that KSHV also utilizes the 12-transmembrane transporter protein xCT for entry into adherent cells (Kaleeba and Berger, 2006). The xCT molecule is part of the cell surface 125 kDa disulfide linked heterodimeric membrane glycoprotein CD98 (4F2 antigen) complex containing a common glycosylated heavy chain (80kDa) and a group of 45 kDa light chains. The xCT molecule involved in glutamate/cystine exchange is one of the light chain (Kaleeba and Berger, 2006). Expression of recombinant xCT rendered otherwise not susceptible target cells permissive for both KSHV cell fusion and virion entry. Antibodies against xCT blocked KSHV fusion and entry with naturally permissive target cells such as the adherent target cells of human and nonhuman cell types. However, xCT mRNA was not detected in human CD19 primary B cells isolated from fresh peripheral blood mononuclear cells (Kaleeba and Berger, 2006). These studies further suggest that like EBV, KSHV may possess alternative receptor(s) in adherent and non-adherent cells and other molecules besides xCT may be involved in infection of B cells. It is interesting to note that the CD98 complex usually associates with $\beta 1$ integrin and has been shown to be involved in membrane clustering and $\beta 1$ integrin-mediated signal cascades (Fenczik *et al*,. 2001; Feral *et al.*, 2005).

Cell surface signaling during entry

The interactions of eukaryotic cells with their extracellular environments are largely mediated by ligand-induced signaling molecules exposed at the cell surface. The ensuing multitudes of biological processes are mediated by highly inter-linked networks of signaling pathways. Ligand mimicry is an opportunistic mechanism by which microbes, including viruses, subvert host signaling molecules for their benefit (Virji, 1996). By evolving to use cell surface molecules for attachment or entry into a cell, viruses have also evolved to take advantage of the events triggered by signaling to facilitate intracellular transport, to manipulate cell defense mechanisms, or to induce the pattern of cellular gene expression that is most conducive to establishment of latent or productive infection. Understanding virus induced signaling and its consequence is emerging as an important area of virology.

Signaling by EBV during the early stages of infection

The B cell receptor for EBV, CR2, can function as a signal transducer both independently and as a part of a signal transduction complex. This complex, composed of CR2, CD19 and CD81, modifies cell surface immunoglobulin-mediated signaling. CR2-transduced signaling is not required for infection of tumor derived cell lines by EBV (Martin *et al.*, 1994), which suggests that it is not required for intracellular transport or uncoating of virus, but several studies suggest that it may be critical for transformation of a resting B cell. Binding of EBV to CR2 stimulates capping of both CR2 and immunoglobulin and leads to increased blast formation, cell adhesion, surface CD23 expression and increased RNA synthesis (Gordon *et al.*, 1986; Tanner and Tosato, 1992). Interleukin 6, which is a paracrine or autocrine growth factor for EBV immortalized B cells (Tanner and Tosato, 1992), is activated by purified gp350/220 by a pathway that is sensitive to inhibitors of protein kinase C and tyrosine kinases and probably occurs as a result of downstream activity of NFκB (D'Addario *et al.*, 2001). Activation of NFκB increases transcription from the EBV Wp promoter which is responsible for initiation of latent gene expression and inhibition of NFκB inhibits transformation (Sugano *et al.*, 1997). Interaction of gp350/220 with CR2 also induces tyrosine phosphorylation of CD19 and activation of phosphatidylinositol 3-kinase (PI 3-K). Inhibition of these pathways inhibits expression of essential transforming genes (Sinclair and Farrell, 1995).

It is unlikely that signal transduction by EBV structural components and cell proteins is limited to the interaction between gp350/220 and its effects on gene expression, but

potential effects of other virus proteins have yet to be studied. The interactions between gp42 and HLA class II and between the BMRF2 protein and integrins, which, as discussed in detail below, play important roles in HHV-8 infection, appear to be very promising avenues for future exploration.

Signaling by KSHV during the early stages of infection

The integrins with which KSHV gB interacts are part of a large family of heterodimeric receptors containing noncovalently associated transmembrane α and β glycoprotein subunits (Giancotti, 2000; Giancotti and Ruoslahti, 1999; Sastry and Burridge, 2000). There are 17 α and 9 β subunits, generating more than 24 known combinations of αβ cell surface receptors. Each cell expresses several combinations of αβ integrins, and each αβ combination has its own binding specificity and signaling properties (Giancotti, 2000; Giancotti and Ruoslahti, 1999; Sastry and Burridge, 2000). Integrin interactions with ECM proteins provide robust signals for host-cell gene expression and mediate a variety of cell functions such as activation of cytoskeleton elements, endocytosis, attachment, cell cycle progression, cell growth, apoptosis, and differentiation (Giancotti, 2000; Giancotti and Ruoslahti, 1999; Sastry and Burridge, 2000). FAK is a non-receptor protein-tyrosine kinase that localizes in focal adhesions with vinculin, and FAK activation is the first step necessary for the outside–in signaling of integrins (Calderwood *et al.*, 2000; Giancotti, 2000; Sastry and Burridge, 2000). Within 5 minutes of infection, KSHV induces the integrin-mediated activation of FAK in endothelial and fibroblast cells, and co-localizes with the focal adhesion component vinculin (Akula *et al.*, 2002). Soluble gB, but not soluble gpK8.1A, induces FAK, which also colocalizes with pakillin (Wang *et al.*, 2003). FAK activation is inhibited by the pre-incubation of virus or gB with soluble α3β1 integrin or anti-gB antibodies, and is not activated by a soluble form of gB in which the RGD sequence had been mutated (Akula *et al.*, 2002; Wang *et al.*, 2003). The ability of anti-integrin antibodies and soluble integrin to neutralize virus infection without affecting virus entry suggests that integrin and the associated signaling pathways have a role to play in KSHV entry and infection of target cells.

Studies with FAK knockout mouse fibroblasts Du3 ($FAK^{-/-}$) and parental Du17 ($FAK^{+/+}$) cells confirm that FAK plays a key role in HHV-8 infection (Naranatt *et al.*, 2003). Since activation of FAK is central to many paradigms of outside-in signaling by integrins, actin assembly, and endocytosis, KSHV may be taking advantage of these signaling pathways both to promote entry and to produce a cellular state that facilitates infection.

KSHV induced the phosphorylation of FAK in FAK-positive Du17 mouse embryonic fibroblasts early during infection. The absence of FAK in Du3 ($FAK^{-/-}$) cells resulted in about 70% reduction in the internalization of KSHV DNA, suggesting that FAK plays a role in KSHV entry. Expression of FAK in Du3 ($FAK^{-/-}$) cells via an adenovirus vector augmented the internalization of viral DNA. Expression of the FAK dominant-negative mutant FAK-related non kinase (FRNK) in Du17 cells significantly reduced the entry of virus. Reduced quantity of virus entry in Du3 cells, delivery of viral DNA to the infected cell nuclei (Krishnan *et al.*, 2006), and expression of KSHV genes suggested that in the absence of FAK, another molecule(s) may be partially compensating for FAK function. Infection of Du3 cells induced the phosphorylation of the FAK-related proline-rich tyrosine kinase (Pyk2) molecule, which has been shown to complement some of the functions of FAK. Expression of an autophosphorylation site mutant of Pyk2 in which Y402 is mutated to F (F402 Pyk2) reduced viral entry in Du3 cells, suggesting that Pyk2 facilitates viral entry moderately in the absence of FAK. These results suggest a critical role for KSHV infection-induced FAK in the internalization of viral DNA into target cells (Krishnan *et al.*, 2006). One of the important downstream effectors of FAK that is activated directly or through Src kinase via Ras is PI 3-K, a member of a family of lipid kinases (Giancotti and Ruoslahti, 1999; Sastry and Burridge, 2000) that act as second messengers for a number of cell functions including the activation of Rho-GTPases and anti-apoptotic pathway Akt molecule (Giancotti and Ruoslahti, 1999; Sastry and Burridge, 2000). KSHV induces PI 3-K within 5 min of infection which decreased after 15 min (Naranatt *et al.*, 2003). The response can be inhibited by pre-incubating KSHV with integrin and by the PI 3-K inhibitors wortmannin and LY294002. Another hallmark of integrin interaction with ligands is the reorganization and remodeling of actin cytoskeleton. This is controlled by the Rho family of small GTPases, such as Rho, Rac, and Cdc42, and the morphological changes induced by Rho, Rac and Cdc42 activation are downstream effects of PI 3-K activation (Hall and Nobes, 2000). Immediately following KSHV infection, target cells exhibit morphological changes and cytoskeletal rearrangements such as filopodia, lamellipodia and stress fibers. This together with the phosphorylation of PI 3-K by KSHV at early time infection suggests the induction of RHo-GTPases and the associated signal pathways (Naranatt *et al.*, 2003).

FAK represents a point of convergence from activated integrins and initiates a cascade of intracellular signals that eventually activate the mitogen activated protein kinase (MAPK) pathways (Giancotti and Ruoslahti, 1999;

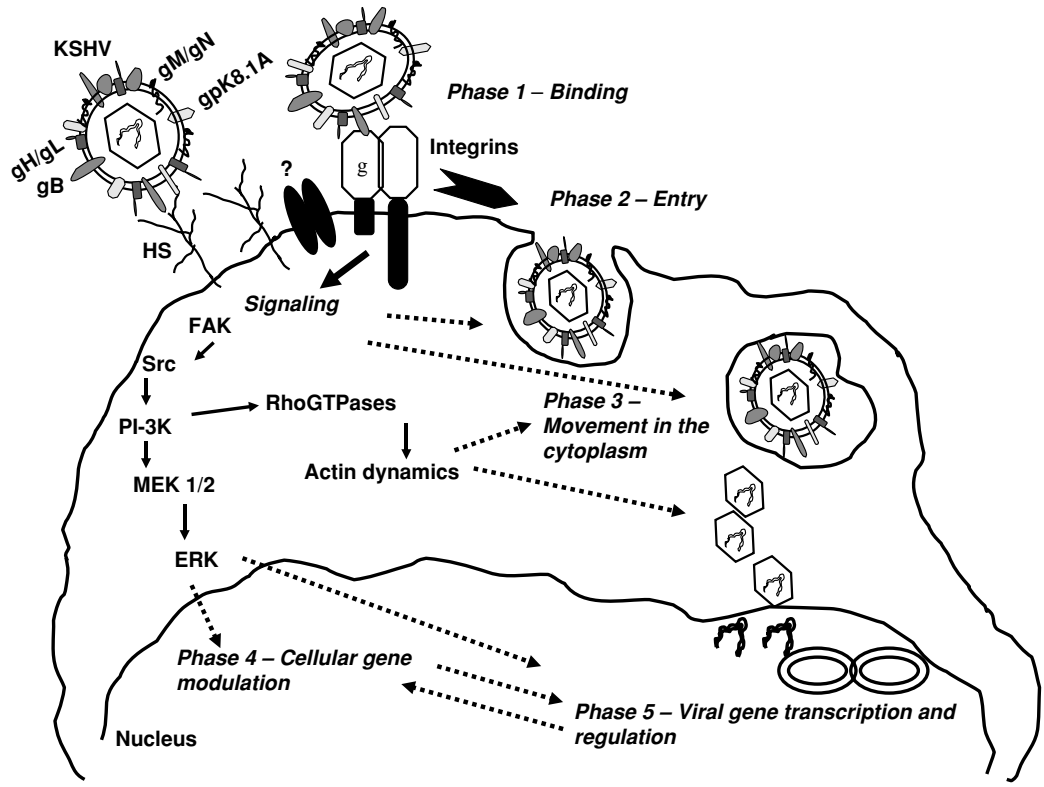

Fig. 23.3. Model for early events of KSHV infection of target cells. Early events of KSHV infection are depicted in overlapping dynamic phases. In phase 1, KSHV binds to the cell surface via its interactions with heparan sulfate proteoglycans (HSPGs) and integrins and possibly to other yet to be identified molecule(s). In phase 2, virus enters into the target cells, overlapping with the induction of host cell signal pathways. In phase 3, viral capsid/tegument moves in the cytoplasm, probably facilitated by the induced signal pathways, and probably overlaps with phase 4, the induced host cell gene transcription and expression. In phase 5, viral DNA enters into the nucleus followed by the viral gene expression. Solid arrows depict the KSHV-induced host cell signaling events that have been so far characterized. The dotted lines depict the potential stages of virus entry and infection at which the induced signaling events may have a role based on the known functions of these signaling events.

Sastry and Burridge, 2000). MAPK pathways exist in all eukaryotes and control fundamental cellular processes such as proliferation, differentiation, survival and apoptosis (Giancotti and Ruoslahti, 1999; Sastry and Burridge, 2000). As early as 5 minutes postinfection, KSHV activates the MEK (MAPK/ERK kinase) and extracellular-signal-regulated kinase (ERK) (Naranatt *et al.*, 2003) (Fig. 23.3). Focal adhesion components PI 3-K and protein kinase C-ζ (PKC-ζ) are recruited as upstream mediators of the HHV-8 induced ERK pathway.

Antibodies to KSHV-gB that neutralize infection and soluble α3β1 integrin inhibit the virus-induced ERK signaling pathways. Early kinetics of the cellular signaling pathway and its activation by UV-inactivated KSHV suggest a role for virus binding or entry, but not viral gene expression, in this induction. Studies with human α3 integrin transfected CHO cells, and FAK negative mouse DU 3 cells suggest that the α3β1 integrin and FAK play critical roles in the KSHV mediated signal induction (Naranatt *et al.*, 2003). Inhibitors specific for PI 3-K, PKC-ζ, MEK and ERK significantly reduce virus infectivity without affecting virus binding to the target cells. Examination of entry of viral DNA supports a role for PI 3-K in KSHV entry and a role for PKC-ζ, MEK and ERK at a stage after entry (Naranatt *et al.*, 2003). PI 3-K is involved in the activation of Rho-GTPases. These in turn are critical for the activation of Rac, Rho, Cdc42 and Rab5 which are involved in the modulation of actin dynamics, formation of endocytic vesicles and the fission of endocytic vesicles. Furthermore, viral capsid movement in the cytoplasm probably depends upon the microfilaments and microtubules, which are controlled by the RhoGTPases. Since KSHV induces the RhoGTPases, it is reasonable to speculate that these inductions serve a vital role in the infectious process. The interaction of KSHV with

cells induces the polymerization of cortical actin filaments (Naranatt *et al.*, 2003). Further detailed analyses are essential to decipher the link between these pathways and their potential roles not only in KSHV entry into target cells but also the release and movement of capsids in the cytoplasm and delivery of viral DNA into the nucleus.

Besides playing an important role in the entry of viral nucleic acid into the nucleus of the infected cells, KSHV interactions with HS, integrins and other host cell surface molecules may also dictate the outcome of an infection by creating an appropriate intracellular environment facilitating infection. For example, there are many obstacles that viruses have to overcome during the early and late stages of infection of target cells in the host. They include external threats to infected cells from the innate and adaptive immune systems as well as internal obstacles such as transcriptional blocks and cellular apoptosis that may be triggered by virus binding and entry. To establish a successful infection, herpesviruses must have developed many ways to manipulate and overcome these obstacles early during infection. In this respect, stimulation of PI 3-K by KSHV, which may influence the Akt induced anti-apoptotic pathway, and modulation of interferon response factors by the virion associated tegument protein ORF 45 protein (Zhu and Yuan, 2003) are of considerable interest.

Cytoplasmic trafficking, delivery of viral genome into the nucleus

After release into the cytoplasm, the EBV and KSHV capsid/tegument must traffic through the cell in order for viral DNA to be delivered into the nucleus. HSV-1 utilizes dynein motors and microtubules for this purpose and the activation by KSHV of RhoGTPases, which are important to control of microtubules, is consistent with a similar mechanism of transport for this virus.

Similar to HSV, KSHV utilizes the dynein motors in the cytoplasmic trafficking and delivery of viral DNA to the nucleus (Narranat *et al.*, 2005). Microtubules play important roles in KSHV infection since depolymerization of microtubules even though did not affect KSHV binding and internalization, it inhibited the nuclear delivery of viral DNA and infection (Narranat *et al.*, 2005). The interesting aspect is that KSHV induced the acetylation of microtubules, an essential step for the microtubule aggregation, which are mediated by the host cell pre-existing signals induced by KSHV binding an entry steps. The inactivation of Rho GTPases by Clostridium difficile toxin B significantly reduced the microtubular acetylation and the delivery of viral DNA to the nucleus (Narranat *et al.*, 2005). Activation of Rho GTPases by *Escherichia coli* cytotoxic necrotizing factor significantly augmented the nuclear delivery of viral DNA. Activation of RhoA-GTP-dependent diaphanous 2 was observed, with no significant activation in the Rac- and Cdc42-dependent PAK1/2 and stathmin molecules. The nuclear delivery of viral DNA increased in cells expressing a constitutively active RhoA mutant and decreased in cells expressing a dominant-negative mutant of RhoA. Like in HSV-1, KSHV capsids colocalized with the microtubules, and the colocalization was abolished by the destabilization of microtubules with nocodazole and by the PI-3K inhibitor affecting the Rho GTPases. These results suggest that KSHV induces Rho GTPases, modulates microtubules and promotes the trafficking of viral capsids and the establishment of infection (Narranat *et al.*, 2005). These studies demonstrated for the first time modulation of the microtubule dynamics by virus-induced host cell signaling pathways to aid in the trafficking of viral DNA to the infected cell nucleus. These data strongly suggest that KSHV manipulates the host cell signaling pathway to create an appropriate intracellular environment that is conducive to the establishment of a successful infection.

No studies of EBV have directly addressed this issue, although the observation that at least in epithelial cells the EBV BMRF2 protein interacts with integrins is provocative (Tugizov *et al.*, 2003). In primary B cells transcription from incoming EBV DNA can be detected within 10–12 h post infection and since circularization of the genome requires host protein synthesis (Sinclair and Farrell, 1995), whereas initiation of transcription from the viral genome does not (Hurley and Thorley-Lawson, 1988), it can be inferred that transcription initiates from the incoming linear genome. Transcription is unaffected by inhibitors of tyrosine kinases and PI 3-K, but synthesis of virus proteins is reduced implying that stimulation of kinases is not required for transport of virus to the nucleus, as it may be for KSHV, but for a later stage in the infection process (Sinclair and Farrell, 1995). Circular episomes have been detected by 16 h post infection and their formation may require that the cell move from G0 to G1 (Alfieri *et al.*, 1991; Hurley and Thorley-Lawson, 1988). De novo protein synthesis is probably necessary for circularization and a cellular protein complex that includes Sp1 and binds to the recombination junctions within the terminal repeats of the genome may be involved in the process (Sun *et al.*, 1997). Early studies have suggested that amplification of the first formed episome does not occur in primary B cells until more than one week after infection (Hurley and Thorley-Lawson, 1988). No similar studies have yet been reported for KSHV, although the virus is found in episomal form in PEL cells (Ballestas *et al.*, 1999). Clearly much more needs to be known about the events between entry and early transcription from the incoming genome for both KSHV and EBV.

Summary

The importance of EBV and KSHV as oncogenic viruses has appropriately focused efforts on understanding the ways in which they influence cell survival and growth. An unintended consequence has been that information about productive replication of both viruses has been less forthcoming. Recent progress, particularly in understanding the early events that initiate entry, has been promising, but many important gaps remain to be filled. The determinants of tropism remain incompletely defined for both EBV and KSHV and although the perennial problem of how herpesviruses mediate virus cell fusion is not unique to either EBV or KSHV, it is still largely unsolved. The effect of entry on cell signaling pathways is proving to be an extremely fertile area of study, not just for understanding how viruses establish a suitable intracellular milieu for gene expression and DNA replication, but also for understanding how viruses are transported to the nucleus after the cell membrane has crossed. Progress with KSHV has been particularly interesting in this regard. It already appears likely that infection of endothelial cells by KSHV and infection of B cells by EBV may follow different pathways. However, whether this reflects fundamental differences in virus strategy, or adaptation to different host cells will require study of the viruses in more than one of the cell types that each infects. As important as a comprehensive understanding of lytic replication is to understanding the pathogenesis of disease as a whole, for EBV and KSHV it remains a distant goal.

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24

Maintenance and replication during latency

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Introduction

The human gammaherpesviruses Epstein–Barr Virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) are associated with a variety of malignancies involving cells of various lineages.

After primary infection and initial viral propagation in epithelial and lymphoid cells, both viruses establish latency in a subset of CD19 positive B-cells. In this often lifelong asymptomatic stage, in which EBV genomes are detectable primarily in resting memory B-cells, the number of infected cells is extremely low and virus load is tightly controlled by the host cellular and humoral immune response.

Loss of this balance leads to an increase in viral load, which often precedes the onset of malignant diseases. Both tissue involvement and histopathology are highly variable between EBV- and KSHV-associated malignancies and involve either lymphoid (Burkitt's lymphoma and primary effusion lymphoma), epithelial (nasopharyngeal carcinoma), or endothelial (Kaposi's sarcoma) tissues.

However, common to all gammaherpesvirus-associated tumors is that the majority of tumor cells are latently infected, and harbor extrachromosomal circularized viral genomes called episomes that are replicated and segregated by the host cellular replication machinery indefinitely. This ability to maintain long-term latent infection in quiescent and proliferating cells may be a defining property shared by both the lymphocryptoviruses (LCV, represented by EBV) and the rhadinoviruses (RDV, represented by KSHV).

This chapter aims to summarize our current understanding of the underlying mechanisms by which EBV and KSHV achieve long-term episomal maintenance in latently infected cells, which conceptually can be viewed as a two step process: replication of the viral genome and faithful segregation to daughter cells (Fig. 24.1).

Before going into the details about viral DNA replication, episomal maintenance and their *cis*- and *trans*-requirements, it is important to note that much less is known about the molecular events that precede the establishment of stable latency after primary infection. Initially, KSHV, like most herpesvirus virions, attaches to heparin sulfate prior to interactions with its receptor. On B-cells KSHV binds to members of the alpha integrin family which may serve as receptor or coreceptor (Akula *et al.*, 2001, 2002). However, EBV does not encode a heparin binding protein and directly binds to the high affinity receptor CD21 which is highly expressed on B-cells (Ahearn *et al.*, 1988; Li *et al.*, 1992). Attachment, binding and internalization, which for both viruses are believed to be dependent on endocytosis, are discussed in detail in Chapter 23. Virion binding to cell surface receptors also triggers signal transduction pathways, which may facilitate viral entry. The concurrent steps: transport of capsids through the cytoplasm and delivery of the linear viral genome to the nucleus have not been studied in great detail for either EBV, or KSHV. It is believed that circularization of viral genomes occurs early after infection prior or concurrent with the onset of viral mRNA synthesis (Hurley and Thorley-Lawson, 1988; Alfieri *et al.*, 1991). Recent studies on KSHV show that DNA is detectable in infected cells as early as 5 min post-inoculation. Furthermore, first viral transcripts are detectable by 30 min post-infection. Surprisingly, one study on KSHV found that many of the genes expressed after *de novo* infection were early lytic genes rather than latency-associated genes. However, after latency-associated genes were detectable, a marked down-regulation of lytic gene expression was noted (Naranatt *et al.*, 2004). This new finding suggests that early lytic genes contribute the establishment of latency by providing auxiliary functions such as perturbing immune recognition of infected cells. The

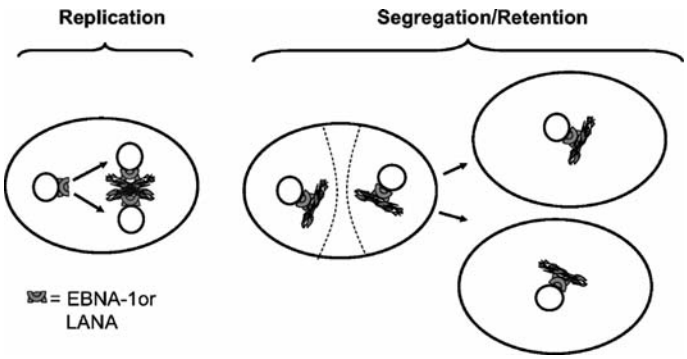

Fig. 24.1. Plasmid maintenance model for EBV and KSHV. EBNA1 and LANA tether viral genomes to cellular chromosomes. This tethering can occur through numerous chromosome-associated proteins and appears to be sufficient for stable plasmid maintenance.

question about how latency is established is also discussed in Chapter 23 which is specifically focused on early events after infection. The focus of this chapter, however, is how the viral episome is maintained in cells in which latency has been established.

During latency, viral gene expression is limited to a small subset of genes dedicated to viral genome maintenance and host-cell accommodation. Although many genes required for lytic replication and virion structure are highly conserved among the herpesvirus family, those genes required for the establishment and maintenance of latency are quite distinct even within the gammaherpesviruses. EBV encodes a total of eight different latency-associated genes whose expression differences among lymphoid malignancies gave rise to the classification of three latency programs. While some Burkitt's lymphomas express only a single latent antigen (Epstein–Barr virus nuclear antigen 1, EBNA1), rare immunoblastic lymphomas may express all EBNAs in addition to the latent membrane proteins (LMP 1 and 2). For KSHV, gene expression analysis showed that tumor cells in KS lesions and PEL-derived cell lines also express only a small number of genes. Although viral gene expression during latency may differ between KS and PEL, PEL-derived cell lines have been instrumental for the identification and characterization of so far five KSHV latency-associated genes. These are the latency-associated nuclear antigens (LANA and LANA2), vCyclin, vFLIP, and Kaposin A and B.

With the exception of LANA and EBNA1, which both play an important role in viral DNA replication and genome maintenance during latency, there is little conservation between the latency-associated genes of lymphocryptoviruses and rhadinoviruses. The functions and roles of these gene products in the biology of KSHV and EBV will be discussed elsewhere in this volume and have recently been subject to a number of excellent review articles (Boshoff and Weiss, 2002; Herndier and Ganem, 2001).

Both LCV and RDV family members encode origin binding proteins (EBNA1 and LANA) and possess genetic elements (origins) dedicated to viral genome maintenance and replication during latent infection in proliferating cells. The specific protein interactions and detailed mechanisms used by LCV and RDV have evolutionarily diverged, but many of the underlying strategies of viral persistence are quite similar. A review of the properties of the latent viral genome and the mechanisms of DNA replication, maintenance, and segregation during latency should highlight important differences and essential similarities among the gammaherpesviruses.

EBV (Lymphocryptovirus)

A number of excellent reviews address the biology of EBV replication and plasmid maintenance during latency (Leight and Sugden, 2000; Sugden and Leight, 2001). Most of this section will focus on the molecular biology of Epstein–Barr nuclear antigen 1 (EBNA1) and its interactions with the origin of plasmid replication (*OriP*). It is this interaction that is primarily responsible for EBV genome stability during latency. However, other aspects of the viral chromosome will be considered, including the importance of DNA methylation status, chromatin organization, and nuclear matrix attachment.

Properties of the episomal latent viral genome

The prototypical latent gammaherpesvirus exists as multicopy (5–100) covalently closed circular genomes that can be separated from chromosomal DNA by density gradient centrifugation (Adams and Lindahl, 1975; Nonoyama and Tanaka, 1975) and by gel electrophoresis using the Gardella gel system (Gardella *et al.*, 1984). However, integrated forms of the virus can be detected, and numerous cell lines have been described that have complete and partial EBV genomes integrated into the cellular chromosome (Adams and Lindahl, 1975; Nonoyama and Tanaka, 1975; Andersson-Anvret and Lindahl, 1978; Lindahl *et al.*, 1978; Koliais, 1979; Anvret *et al.*, 1984; Lawrence *et al.*, 1988; Popescu *et al.*, 1993; Kripalani-Joshi and Law, 1994; Wuu *et al.*, 1996; Ohshima *et al.*, 1998; Chang *et al.*, 2002). In the prototypical latent infection, EBV circularizes rapidly after

initial infection through an annealing event at the terminal repeats (Kintner and Sugden, 1979; Laux *et al.*, 1989; Cheung *et al.*, 1993; Zimmermann and Hammerschmidt, 1995). The circular form is thought to be essential for reactivation of the lytic cycle and production of progeny virus. Circularization is also required for the expression of the terminal repeat transcript LMP2a that may contribute to the stability of the latent state by inhibiting B-cell receptor mediated reactivation signals (Laux *et al.*, 1989; Miller *et al.*, 1994).

Chromatin organization of the latent episome

Packaging of cellular DNA into nucleosomal chromatin is essential for its protection and stability throughout much of the cell cycle. This holds true for viral episomal DNA, as well. During latency, the viral episome is associated with nucleosomes in arrays that are indistinguishable from bulk cellular DNA (Shaw *et al.*, 1979; Dyson and Farrell, 1985). Micrococcal nuclease laddering assays revealed that the majority of the EBV latent genome is organized in nucleosome arrays (Dyson and Farrell, 1985), with the exception of the region covering *OriP* (Wensing *et al.*, 2001). Nucleosomes were also disorganized in the adjacent sequences encompassing the EBERs, which are two genes constitutively transcribed by RNA polymerase III during most forms of latency (Sexton and Pagano, 1989; Wensing *et al.*, 2001). Consistent with these findings, biochemical studies revealed that EBNA1 can displace a mononucleosome positioned over an EBNA1-binding site in vitro, suggesting that EBNA1 binding to *OriP* is sufficient for displacing an ordered array of nucleosomes from this region of the latent genome (Avolio-Hunter *et al.*, 2001).

In contrast to the latent genome, EBV DNA found in the virion or after lytic replication appears to be nucleosome free (Shaw *et al.*, 1979; Dyson and Farrell, 1985). Nucleosome assembly on newly synthesized lytic DNA might inhibit DNA insertion into the nucleocapsid, and thus might inhibit infectious virion production. It is not clear what mechanism prevents nucleosome assembly during lytic infection; nucleosome assembly on newly synthesized cellular DNA is coupled to DNA replication proteins, and these cellular enzymes do not participate in viral lytic replication. Alternatively, viral encoded proteins may actively prevent nucleosome assembly on the viral genome during lytic replication by an as yet unknown mechanism.

Nucleosome positioning and histone modification may also contribute to the gene regulation during latent infection. Lytic gene promoters are transcriptionally repressed during latent infection. Inhibitors of histone deacetylases, like sodium butyrate and trichostatin A, are potent activators of lytic cycle gene expression (Saemundsen *et al.*, 1980; Jenkins *et al.*, 2000). The positioned nucleosome contributes to transcriptional repression of the immediate early gene, but it is not clear how the nucleosome is positioned in the viral genome. Nucleosome positioning may be affected by at least two mechanisms including nucleosome positioning elements inherent in DNA sequence, and sequence specific binding proteins that recruit nucleosome modifying activities. Histone deacetylases associated with DNA binding proteins MEF2D and RBP-Jk are known to maintain repressive chromatin at the EBV BZLF1(Liu *et al.*, 1997; Speck *et al.*, 1997; Gruffat *et al.*, 2002) and EBNA2 Cp (Hsieh *et al.*, 1999; Alazard *et al.*, 2003) promoters. In addition to restricting gene expression, chromatin structure and histone modifications may protect the viral genome from DNA damage during latency. Nucleosome position and remodeling may also play a role in the regulation of plasmid replication during latency of both EBV and KSHV (Stedman *et al.*, 2004; Zhou *et al.*, 2005).

DNA methylation of latent EBV

DNA methylation is an epigenetic mark associated with transcriptional silence (Bird, 1986; Jones and Wolffe, 1999). The EBV genome is highly methylated during latency (Saemundsen *et al.*, 1983) and there is evidence that virion DNA is also hypermethylated (Diala and Hoffman, 1983). DNA methylase inhibiting drugs, like 5-aza-cytidine, can induce lytic gene expression from latently infected cells (Ben-Sasson and Klein, 1981). DNA methylation has also been found to play a critical role in regulating LMP1 and EBNA2-6 transcription (Ernberg *et al.*, 1989; Masucci *et al.*, 1989; Allday *et al.*, 1990; Hu *et al.*, 1991; Minarovits *et al.*, 1994; Robertson *et al.*, 1996; Robertson and Ambinder, 1997; Schaefer *et al.*, 1997; Falk *et al.*, 1998; Ambinder *et al.*, 1999; Robertson, 2000) and thus contributes to the transition between various latency programs.

Like nucleosome phasing, DNA methylation is not uniform throughout the viral genome. DNA methylation is generally spared from sequence elements of *OriP* (Falk and Ernberg, 1993; Salamon *et al.*, 2001). EBNA1 binding to *OriP* contributes to the sparing of DNA methylation, but the mechanism preventing DNA methylation is not known (Hsieh, 1999; Lin *et al.*, 2000; Salamon *et al.*, 2000). EBNA1 bound to *OriP* can also affect transcription of LMP1p (Gahn and Sugden, 1995) and Cp (Reisman and Sugden, 1986; Puglielli *et al.*, 1996; Nilsson *et al.*, 2001), and it is possible that effects on DNA methylation and chromatin structure

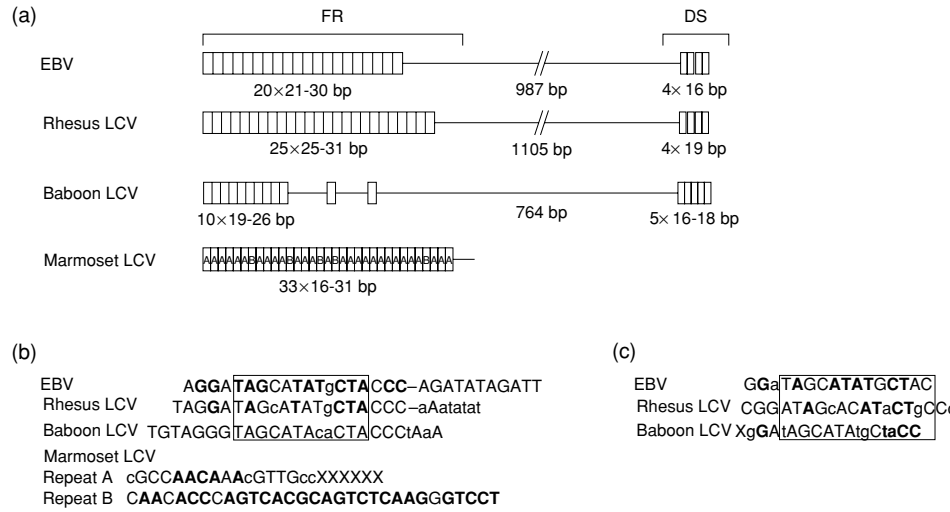

Fig. 24.2. Comparative *OriP* structure of lymphocryptoviruses. (a) Family of repeat (FR) and dyad symmetry elements of human and primate viruses. (b) and (c) Alignment of EBNA1 binding sites within FR and DS elements (courtesy of Rivaillier *et al.* (2002).

at *OriP* contribute to latent cycle transcription. In this respect, *OriP* may be a significant control element determining gene expression patterns in the various types of EBV latency.

Molecular biology of *OriP*

The genetic elements responsible for plasmid maintenance and DNA replication were identified through the elegant experiments of Yates and Sugden (Yates *et al.*, 1984, 1985; Reisman *et al.*, 1985). In these experiments, plasmid subclones of the B95-8 genome were tested for their stable maintenance in EBV positive cell lines and only one region (nucleotides 7000 and 9258 in EBV coordinates) possessed clear plasmid maintenance and DNA replication activity (Yates *et al.*, 1984). This region is referred to as the origin of plasmid replication (*OriP*) and consists of two functional domains, the family of repeats (FR) and the Dyad symmetry (DS) region (Fig. 24.2) (Lupton and Levine, 1985; Reisman *et al.*, 1985; Yates *et al.*, 1985; Chittenden *et al.*, 1989; Wysokenski and Yates, 1989). The Epstein–Barr virus nuclear antigen 1 (EBNA1), which is expressed in most forms of viral latency, binds directly to the 30 bp repeat elements found in FR and DS in *OriP* (Rawlins *et al.*, 1985). EBNA1 is the only viral protein required for episomal maintenance and replication of *OriP*-containing plasmids (Yates and Camiolo, 1988; Wysokenski and Yates, 1989).

OriP consists of at least two functional elements that are separated by a non-essential 1 kb spacer region (Fig. 24.2). The family of repeats (FR) consists of 20 × 30 bp repeats each containing a 16 bp EBNA1 binding site. The DS element consists of four lower affinity EBNA1 binding sites that are organized as two functional pairs. The minimal replicator for *OriP* consists of one functional pair of EBNA1 binding sites spaced precisely 21 nucleotides from the center of each site (Yates *et al.*, 2000). The DS element of EBV consists of two minimal replicators, and mutagenesis shows that at least one intact pair is essential for replication function (Harrison *et al.*, 1994; Koons *et al.*, 2001). DS also consists of three nine base pair repeat elements, referred to as nonamer repeats, which are positioned adjacent to each pair of EBNA1 sites (Niller *et al.*, 1995; Vogel *et al.*, 1998). Mutations of the nonamer sites have 2–5 fold effects on replication and plasmid maintenance, but appear not to be absolutely essential for minimal replicator activity (Vogel *et al.*, 1998; Yates *et al.*, 2000; Koons *et al.*, 2001; Deng *et al.*, 2002, 2003). The nonamer sites are identical to telomere repeats, and were found to interact directly with the telomere repeat binding factors TRF1 and TRF2 (Deng *et al.*, 2002).

An *OriP*-like structure is conserved in sequence and position in all lymphocryptovirus family members, although there is considerable variation in the number, spacing, and sequence of EBNA1 binding sites (Rivailler *et al.*, 2002) (Fig. 24.2). The rhesus EBV (CalHV-3) contains an unusual family of repeats that consists of two repetitive sequences that diverge considerably from consensus EBNA1 sites. This observation is surprising since the DNA binding domain of the rhesus EBNA1 protein is strongly conserved.

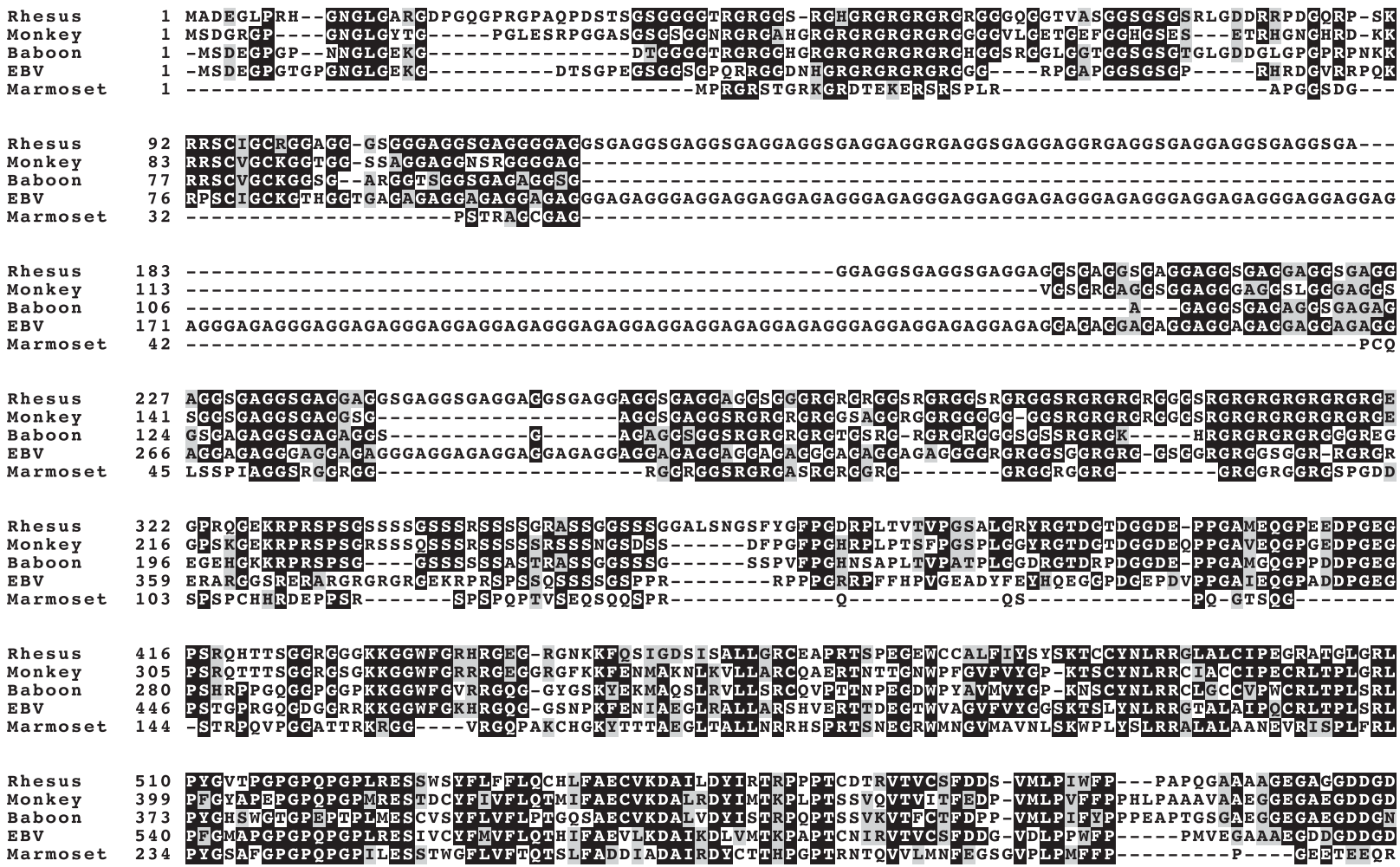

Fig. 24.3. EBNA1 proteins are highly conserved among lymphocryptoviruses. Amino acid alignment demonstrating that the N- and C-terminus of EBNA1 from all five viruses are conserved. Only Rhesus and EBV contain the immune modulatory GA repeats.

Interestingly, no apparent DS element could be identified at the correct position in the rhesus genome (Rivailler *et al.*, 2002). A recent study has shown that the 14 bp spacing (found in EBV) is optimal for plasmid maintenance, while the 10 bp spacing (found in HVP) functions better in transcription enhancement (Hebner *et al.*, 2003). Earlier studies indicated that at least 7-8 EBNA1 binding sites were required for FR function in plasmid maintenance assays, and this seems to vary depending on the spacing of these sites (Lupton and Levine, 1985; Chittenden *et al.*, 1989; Hebner *et al.*, 2003). The spacing between EBNA1 sites in the DS appear to be more highly conserved, and these spacing constraints are likely to be critical for replication initiation function (Bashaw and Yates, 2001)

Properties of EBNA1

All of the known members of the lymphocryptovirus family have a protein with homology to EBNA1, including those found in the rhesus (cercopithicine HV 15), marmoset (callitrichine herpesvirus 3), baboon (herpesvirus papio), and squirrel monkey (cynomolgus herpesvirus A4) (Fig. 24.3). Human Epstein–Barr virus EBNA1 has been most intensively studied, and is necessary and sufficient for *OriP*-dependent DNA replication and plasmid maintenance (Yates *et al.*, 1984, 1985). EBNA1 deletion mutants fail to establish episomal latent infection (Lee *et al.*, 1999) indicating that EBNA1 is required in the context of the intact genome. In addition to its role in DNA replication, EBNA1 can activate transcription from a synthetic plasmid containing multiple EBNA1 binding sites upstream of simple viral promoter (Wysokenski and Yates, 1989). *OriP* bound EBNA1 can function as a transcriptional enhancer for the LMP1 promoter and EBNA2–6 Cp promoter 2–10 kilobase pair away. EBNA1 may also possess additional activities associated with B-cell immortalization since EBNA1 deletion leads to a dramatic decrease in immortalization efficiency (Humme *et al.*, 2003) and its overexpression inhibits apoptosis (Kennedy *et al.*, 2003).

EBNA1 can be divided into several functional domains, with the C-terminal domain (aa 450–620) encoding the DNA binding and dimerization activity essential for interaction with DS, FR, and BamHI Q binding sites (Fig. 24.4) (Rawlins *et al.*, 1985; Ambinder *et al.*, 1990, 1991; Chen *et al.*, 1993, 1994; Goldsmith *et al.*, 1993; Ceccarelli and Frappier, 2000; Cruickshank *et al.*, 2000; Wu *et al.*, 2002). This domain has been crystallized with and without DNA (Fig. 24.5) (Bochkarev *et al.*, 1995, 1996), and shows a remarkable similarity to the structural fold of the DNA binding domain of bovine papilloma virus E2 protein, suggesting that these viral proteins share common ancestral precursor involved in plasmid-based replication. The papillomavirus E2 protein binds the papillomavirus DNA replication origin and helps to load the viral encoded helicase, E1 (Edwards *et al.*, 1998). E2 also has important function in papillomavirus transcription regulation and, like EBNA1, is involved in both positive and negative regulation of gene expression (Desaintes and Demeret, 1996).

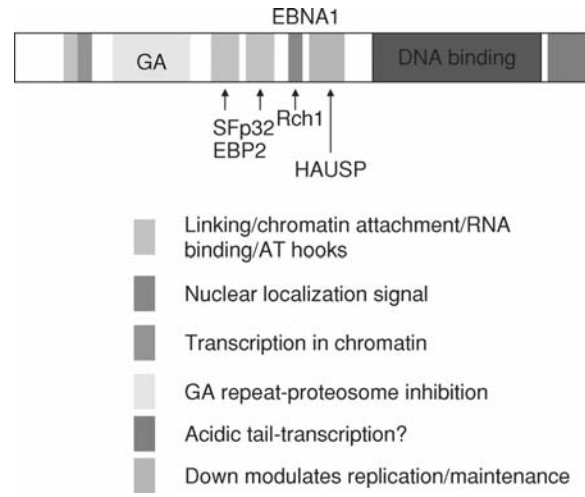

Fig. 24.4. Functional protein domains of EBNA1. Amino acid residues are indicated for the boundaries of protein domains for DNA binding, dimerization, chromosome binding sites (CBS), nuclear localization (NLS), or protein–protein interactions (as indicated). (See color plate section.)

C-terminal DNA binding domain

A detailed analysis of the X-ray crystal structure of the EBNA1 DNA binding domain reveals a fascinating and unique DNA interaction interface, with extensive protein DNA contacts in both the major and minor grooves of the DNA helix (Fig. 24.5) (Bochkarev *et al.*, 1996). The structures reveal that EBNA1 can introduce a strong bend in bound DNA, and provides some insight into the likely contacts between critically phased dimer binding sites in the DS minimal replicon. EBNA1 has been shown to induce permanganate sensitivity upon thymine residues of one strand on the two outermost binding sites in DS (sites 1 and 4) (Hearing *et al.*, 1992). Structural studies revealed that permanganate sensitivity is not a result of duplex breathing caused by single stranded DNA formation, but rather resulted from an unusual helical distortion of a thymine residue on one strand of the EBNA1 binding sites in DS (Summers *et al.*, 1997; Bochkarev *et al.*, 1998). The amino acid residues 461 to 469 that make contact with the minor groove of the EBNA1 binding site were shown to be essential for inducing the permanganate sensitivity (Summers *et al.*,

1997). The functional significance of this distorted thymine structure on DNA replication function remains unclear.

N- terminal domain linking activity

The amino terminal domains of EBNA1 possess genetically distinct activities essential for DNA replication, plasmid maintenance and transcription (Yates and Camiolo, 1988; Wu *et al.*, 2002). Multiple regions amino terminal to the DNA binding domain contribute to DNA replication and plasmid maintenance activity (Fig. 24.4). The amino terminal regions of EBNA1 have no obvious sequence homology to other known proteins, but do contain three RGG-rich domains that were first recognized for their similarity to RGG-elements found in hnRNP RNA binding proteins (Burd and Dreyfuss, 1994). The RGG motifs (aa 34–56, and 339–377) were found to confer some RNA binding ability on EBNA1, but the biological significance of EBNA1 RNA binding has not been elucidated (Snudden *et al.*, 1994). These RGG-rich regions in the amino terminal domain also confer intramolecular "linking" between EBNA1-DNA complexes as revealed by the formation of large non-resolved complexes in electrophoresis mobility shift assay (EMSA) (Frappier *et al.*, 1994; Mackey *et al.*, 1995). Electron microscopy revealed that EBNA1 can induce DNA looping between FR and DS (Su *et al.*, 1991; Middleton and Sugden, 1992), and the aa 350–361 were shown to be responsible for DNA looping in both electron microscopy studies and ligation enhancement assays (Frappier *et al.*, 1994). The linking domains (aa 40–89, 331–361, and 372–391) were also found to be important for EBNA1 replication and transcription activity (Mackey and Sugden, 1999). The precise mechanistic contribution of these RGG-rich linking domains remains unclear, but are likely to be involved in the protein-protein interactions and metaphase chromosome binding properties of EBNA1.

Metaphase chromosome attachment

EBNA1 can also associate with metaphase chromosomes through amino acids that appear to overlap the linking domains, and are essential for plasmid maintenance function (Marechal *et al.*, 1999). EBNA1 attachment to metaphase chromosome has been mapped to three domains that correlate well with the ability of EBNA1 to confer plasmid maintenance (Marechal *et al.*, 1999; Kanda *et al.*, 2001; Wu *et al.*, 2002). EBNA1 has also been shown to colocalize with DNA replication compartments even in the absence of viral DNA (Ito and Yanagi, 2003), suggesting that the association with interphase chromatin is independent of viral DNA sequences. Intriguingly, substitution of the amino terminal domain of EBNA1 with the HMG I/Y or histone H1 chromatin binding folds was sufficient to reconstitute efficient plasmid maintenance (Hung *et al.*, 2001),

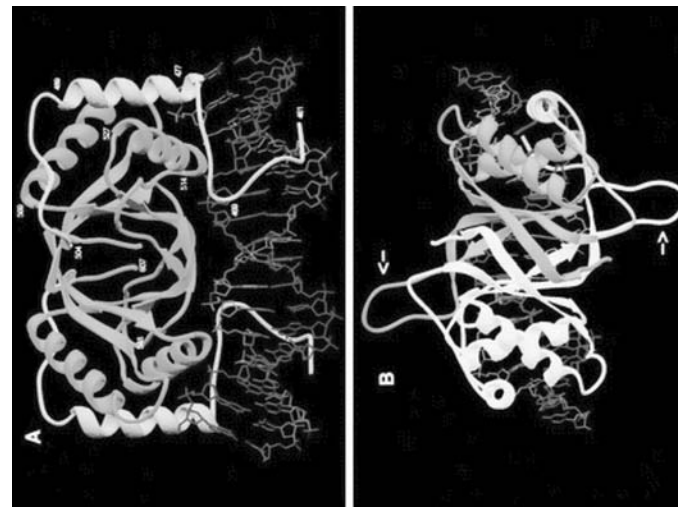

Fig. 24.5. X-ray crystal structure of EBNA1 dimer bound to a consensus DNA recognition site (courtesy of Bocharev *et al.*, *Cell* in press). (a) Ribbon diagram showing the core domain (residues 504–607) from each monomer, in blue. Flanking domains are shown in yellow. (b) View down the non-crystallographic axis showing one monomer in white and the other in the same color scheme as used in (a). Proline loops are indicated by arrows. (See color plate section.)

suggesting that chromosome attachment is both necessary and sufficient for plasmid maintenance. One recent finding indicates that the chromosomal attachment sites of EBNA1 have AT-hook activity, and can bind to AT-rich DNA (Sears *et al.*, 2003, 2004). This suggests that the linking-activity involves weak DNA binding through the amino terminal domain of EBNA1. These data would also account for why this region of EBNA1 can be substituted with HMG I/Y and histone H1, which have AT-hook domains and DNA binding activity.

Transcription regulation

The transcription regulatory properties of EBNA1 have also been investigated. As mentioned above, EBNA1 can stimulate transcription of Cp and LMP1 during latency (Sugden and Warren, 1989; Wysokenski and Yates, 1989; Puglielli *et al.*, 1996; Nilsson *et al.*, 2001). The amino terminal domain of EBNA1 is required for this activity, and can be distinguished from the DNA replication and plasmid maintenance properties by mutational analysis (Yates and Camiolo, 1988; Wu *et al.*, 2002). Transcription activation of chromatin integrated templates required the conserved amino terminal sequence (aa 65–89), which was not essential for activation of transient plasmid based genes (Kennedy and Sugden, 2003). EBNA1 can also function as a transcription repressor, and can down-regulate

transcription of its own mRNA initiating at the F promoter (Sung *et al.*, 1994). In this autoregulatory loop, EBNA1 competes with E2F binding sites necessary for the cell cycle regulation of EBNA1. The detailed mechanisms of EBNA1 transcription activation and repression have not yet been characterized.

Proteosome inhibition by GA repeats

EBNA1 also has a variable length ~200 residue glycine-alanine repeat from aa 100 to 325 using the B95-8 strain. Deletion of the GA repeat has no apparent effect on DNA replication, plasmid maintenance, or transcription regulation. However, the GA repeats inhibit ubiquitin-dependent/proteosomal processing of EBNA1 (Levitskaya *et al.*, 1995, 1997). The GA repeats reduce peptide processing and HLA class I presentation, and are responsible for the limited cytotoxic T-cell response to EBNA1 protein in latently infected cells (Blake *et al.*, 1997). The GA repeats can also inhibit proteosome processing on foreign molecules, and may represent a general strategy for viral proteins to escape immune cell recognition (Levitskaya *et al.*, 1997; Leonchiks *et al.*, 2002). In addition to the effect on protein stability, the GA repeats inhibit EBNA1 translational efficiency, thus contributing to the low, but stable EBNA1 protein expression levels in latently infected cells (Yin *et al.*, 2003).

Cellular proteins that interact with EBNA1

Yeast two hybrid assays and biochemical isolation have identified several cellular proteins that interact with EBNA1. The two hybrid assay isolated SFp32/TAP/hyaluronectin (Wang *et al.*, 1997), importins α and β (Fischer *et al.*, 1997; Kim *et al.*, 1997; Ito *et al.*, 2000), and EBP2 (Shire *et al.*, 1999), while biochemical purifications isolated SFp32(Chen *et al.*, 1998; Van Scoy *et al.*, 2000), and HAUSP (Holowaty *et al.*, 2003). SFp32/TAP binds to two independent regions of EBNA1, aa 40–60 and aa 325–376, which overlaps the RGG motifs and linking domains (Wang *et al.*, 1997) (Fig. 24.4). Overexpression of SFp32 inhibited EBNA1 transcription activation (Wang *et al.*, 1997), and interacts with *OriP* by chromatin immunoprecipitation assay (Van Scoy *et al.*, 2000). SFp32 has also been isolated as a binding partner with the herpesvirus simiri ORF73 LANA protein (Hall *et al.*, 2002) and HIV-1 Tat (Fridell *et al.*, 1995; Yu *et al.*, 1995). SFp32 is thought to have a primary function in oxidative phosphorylation at the mitochondrial membrane (Muta *et al.*, 1997), but may also have a role in the nucleus when shuttled with nuclear binding partners, like EBNA1 (Matthews and Russell, 1998).

EBP2 was also isolated in a two hybrid assay (Shire *et al.*, 1999) and the interaction domain was mapped to the aa 325–376 which overlap with the DNA linking activity that is essential for plasmid maintenance. The yeast EBP2 orthologue has a role in ribosome biogenesis, and has a nucleolar localization (Huber *et al.*, 2000). The most compelling evidence for a role of EBP2 has been in the reconstitution of *OriP* plasmid maintenance in budding yeast (Kapoor *et al.*, 2001). In this system, *OriP* confers centromeric plasmid segregation activity only when EBNA1 is coexpressed with human EBP2. EBP2 and EBNA1 colocalize when expressed in yeast and human cell lines, and EBP2 has been shown to bind to metaphase chromosomes (Wu *et al.*, 2000). EBP2 has been proposed to function in mediating EBNA1 interaction with metaphase chromosomes, and provide EBNA1 with a chromosome attachment and plasmid partitioning function required for stable plasmid maintenance (Wu *et al.*, 2000, 2002).

HAUSP/USP7, along with nucleosome assembly protein (Nap1), template activating factor Ib/SET, protein-kinase CKII, and protein arginine methylase PRMT5, were identified by mass spectrometry analysis of EBNA1 associated proteins (Holowaty *et al.*, 2003). HAUSP/USP7 has also been found to associate with HSV-1 ICP0 (Everett *et al.*, 1999) and this binding correlates with ICP0 transcription activation function. Mutations in EBNA1 that disrupt HAUSP/USP7 binding caused a fourfold increase in plasmid maintenance activity of EBNA1, although no change in EBNA1 protein stability could be detected. EBNA1 binding to HAUSP/USP7 did affect the stability of p53 by blocking its binding and deubiquitylation by HAUSP/USP7. These studies are especially exciting since they provide a mechanism for the anti-apoptotic function of EBNA1 during B-cell immortalization (Saridakis *et al.*, 2005). The functional characterization of other EBNA1-associated proteins has not yet been reported.

Cellular proteins that interact with *OriP*

Cellular factors that interact with *OriP* have also been characterized. A cellular factor, OBP-1/Kid, binds to and competes for the EBNA1 binding sites in *OriP* (Wen *et al.*, 1990; Zhang and Nonoyama, 1994). OBP-1/Kid has kinesin-like motor activity and localizes to metaphase chromosomes and the mitotic spindle (Tokai *et al.*, 1996). A specific role for OBP-1/Kid in *OriP* function has not been apparent, especially since OBP-1 competes for EBNA1 and EBNA1 is thought to bind *OriP* constitutively through most of the cell cycle (Hsieh *et al.*, 1993). The region of EBV encompassing *OriP* and the EBERs associates with the nuclear matrix

(Jankelevich *et al.*, 1992), and it is likely that this binding is dependent upon EBNA1.

Telomere repeat binding factors also bind to *OriP* in association with EBNA1 (Deng *et al.*, 2002). These host-cell proteins include TRF1 and TRF2, as well as hRap1, tankyrase, and poly-ADP ribose polymerase (PARP1). Both tankyrase and PARP1 have poly-ADP ribose polymerase activity, and EBNA1 can be poly-ADP ribosylated in vitro. TRF1 and TRF2 have identical binding specificity for the nonamer repeats in DS, but have opposing activities in DNA replication assays (Deng *et al.*, 2003). TRF2 associates with DS throughout most of the cell cycle, while TRF1 binding peaks in G2/M and its overexpression inhibits *OriP* replication (Deng *et al.*, 2003). The precise function of these telomeric factors and their potential role in EBNA1 modification is under investigation.

Mechanism of *OriP*- DNA replication

OriP can function as a plasmid origin of replication, and the initiation site for replication has been mapped by a two-dimensional gel electrophoresis assay to a region within or quite close to DS (Gahn and Schildkraut, 1989). In contrast, DNA replication pauses or arrests at FR, suggesting that the bulk of DNA replication occurs in a rightward direction initiating at DS (Gahn and Schildkraut, 1989). Earlier studies also demonstrated that *OriP* is subject to cell cycle restricted replication using heavy-light and heavy-heavy strand density gradient isolation (Adams, 1987; Yates and Guan, 1991), as well as by using Mbo1 and Dpn1 restriction enzyme analysis (Shirakata *et al.*, 1999; Hirai and Shirakata, 2001). Early studies demonstrated that EBV genomes replicate once and only once in the early S phase of the cell cycle (Adams, 1987). Subsequently, it was shown that plasmids carrying *OriP* also replicate only once per cell division cycle in EBNA1 positive cells (Yates and Guan, 1991).

DNA replication at cellular origins requires the assembly of pre-replication licensing factors that include the origin recognition complex (ORC), the minichromosome maintenance (MCM) complex, CDC6, Geminin, and CDT1 (Bell and Dutta, 2002). Components of the ORC complex were shown to associate with *OriP* in vivo using chromatin immunoprecipitation assays (Chaudhuri *et al.*, 2001; Dhar *et al.*, 2001; Schepers *et al.*, 2001). Further genetic evidence that *OriP* is regulated by cellular origin components was demonstrated by a failure of *OriP* to replicate in cells haploinsufficient for ORC2 (Dhar *et al.*, 2001). Futhermore, EBNA1 coimmunoprecipitates with ORC2 and overexpression of geminin inhibited *OriP* replication (Dhar *et al.*, 2001). EBNA1 can also interact with purified replication protein A, the cellular single stranded DNA binding protein required for stabilizing the replication fork (Zhang *et al.*, 1998). From these studies, it is apparent that cellular proteins involved in the regulation of chromosomal replication also control *OriP* replication (Fig. 24.6). A recent study demonstrated that TRF2 can bind a subunit of ORC, and stimulate OriP replication (Atanasiu *et al.*, 2006). Despite these many protein interactions, the precise mechanism of ORC recruitment and DNA polymerase loading at OriP remain to be elucidated.

Mechanism of viral chromosome replication

In the context of the complete genome (Little and Schildkraut, 1995; Norio and Schildkraut, 2001) or plasmid containing large chromosomal DNA inserts (Krysan *et al.*, 1989; Krysan and Calos, 1991), replication may initiate at locations other than DS. Consistent with this, DS can be deleted from EBV genomes without disrupting episomal maintenance of the viral genome (Norio *et al.*, 2000). An alternative replicator sequence, referred to as Rep*, was identified between nt 9370 and 9668 in the EBV genome (Kirchmaier and Sugden, 1998). The Rep* can partially restore plasmid maintenance and DNA replication activity to FR plasmids lacking DS. Rep* does not contain any detectable EBNA1 binding sites by sequence analysis or by direct DNA binding assays, suggesting cellular initiation factors may bind to this region in the absence of EBNA1.

OriP and EBNA1 suffice for the reconstitution of DNA replication and plasmid maintenance on transfected plasmid DNA. However, in the context of the episomal viral genome, it appears as if *OriP* is only used a fraction of the time to initiate DNA synthesis during latency (Little and Schildkraut, 1995). Single molecule analysis of replicated DNA (SMARD) also indicated that *OriP* was not the primary site for initiation of DNA replication in Raji Burkitt

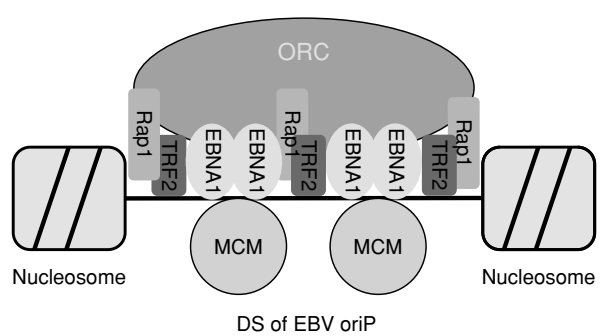

Fig. 24.6. Model for *OriP* of EBV. Indicated are protein–protein and protein DNA interactions that are required for the initiation of DNA synthesis at *OriP*. (See color plate section.)

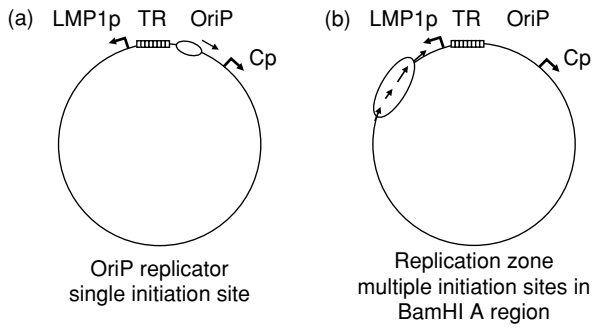

Fig. 24.7. Replication zone versus *OriP*. While in plasmid-based assays DNA synthesis starts at a discrete site near *OriP* (a) in EBV infected cells ori activity is dispersed over a replication zone (b).

lymphoma cell lines, and that the majority of replication initiated from a diffuse zone centered near a PAC1 site in the Bam HI A region (B95-8 coordinates 153, 637) (Little and Schildkraut, 1995; Norio *et al.*, 2000; Norio and Schildkraut, 2001). Initiation of replication in higher eukaryotes may be determined by higher order chromatin domains, rather than discrete replicators, like DS (Gilbert, 2001). EBV appears to have at least two mechanisms for initiating DNA replication, from the discrete DS replicator, or from the broader Bam HI A centered domain of the genome (Fig. 24.7). The possibility that Bam HI A has a replication initiation zone function is particularly intriguing since this region of the genome is transcriptionally active in most forms of EBV latency, and encodes a number of small RNA transcripts (Sadler and Raab-Traub, 1995). The alternative use of replication zones would explain why DS is dispensable for episomal maintenance of the EBV genomes during latency (Norio *et al.*, 2000). It is also likely that the large fragments of cellular DNA that substituted for DS in cosmids also possessed replication initiation zone activity (Krysan *et al.*, 1989; Krysan and Calos, 1991).

Mechanisms of plasmid segregation

Regardless of the flexibility in the site of replication initiation, EBNA1, and presumably FR, are essential for stable episomal maintenance (Lee *et al.*, 1999). EBV lacking EBNA1 can not establish latent episomal genomes (Lee *et al.*, 1999; Humme *et al.*, 2003). Tethering of *OriP* to metaphase chromosomes is thought to be the primary mechanism of plasmid maintenance (Fig. 24.1). The interactions with metaphase chromosome components appears to be highly flexible since substitution of the HMG I/Y and histone H1 chromosome binding regions with EBNA1 linking domains can rescue plasmid maintenance (Hung *et al.*, 2001). Chromosome tethering appears to be mediated by several factors, including the chromatin associated-EBP2 and the AT-hook activity found in the EBNA1 linking domains. Additionally, telomeric factors that bind adjacent to and cooperatively with EBNA1 at DS, contribute to *OriP* plasmid maintenance (Deng *et al.*, 2002). Although the precise role of telomere repeat factors at DS is unclear, recent studies suggest that they contribute to the establishment of a replication and maintenance competent plasmid, perhaps by contributing to the organization of chromatin structure on *OriP*-containing plasmids and facilitating metaphase chromosome attachment.

KSHV (Rhadinovirus)

Shortly after the discovery of KSHV DNA by representational display analysis in KS lesions (Chang *et al.*, 1994), gene expression analysis by *in situ* hybridization and RT-PCR showed that KSHV in KS-lesions are predominantly latently infected (Zhong *et al.*, 1996). Similarly, PEL-derived cell lines are latently infected and, analogous to EBV-infected Burkitt's lymphoma lines, can be induced to enter the lytic replication cycle (Cesarman *et al.*, 1995; Renne *et al.*, 1996a,b). Like BL cell lines for EBV, PEL-derived cell lines have been instrumental for cloning and characterization of the KSHV genome and for the identification of the latency-associated genes.

The KSHV episome in latently infected cells

KSHV episomal viral genomes have been detected by Gardella gel analysis in KS lesions and PEL-derived cell lines (Renne *et al.*, 1996a). Different PEL cell lines contain varying copy numbers (25 to several 100) that are stably maintained over time (Cesarman *et al.*, 1995; Renne *et al.*, 1996b; Cannon *et al.*, 2000). While deletions and duplications have been detected in PEL cells, no integration events have been reported for KSHV. The fact that sodium butyrate, a potent histone deacetylase inhibitor induces lytic replication in PEL cells suggests that nucleosome organization limits lytic cycle gene expression. Lu *et al.* demonstrated that a deacetylated nucleosome positioned over the transcriptional initiation sites of the ORF50 immediate early promoter and its acetylation and remodeling correlated with transcriptional activation and initiation of lytic cycle gene expression (Lu *et al.*, 2003). Similar to EBV, DNA methylation might play a role in the regulation of latency; recent studies suggest methylation of the ORF50 promoter during latency (Chen *et al.*, 2001).

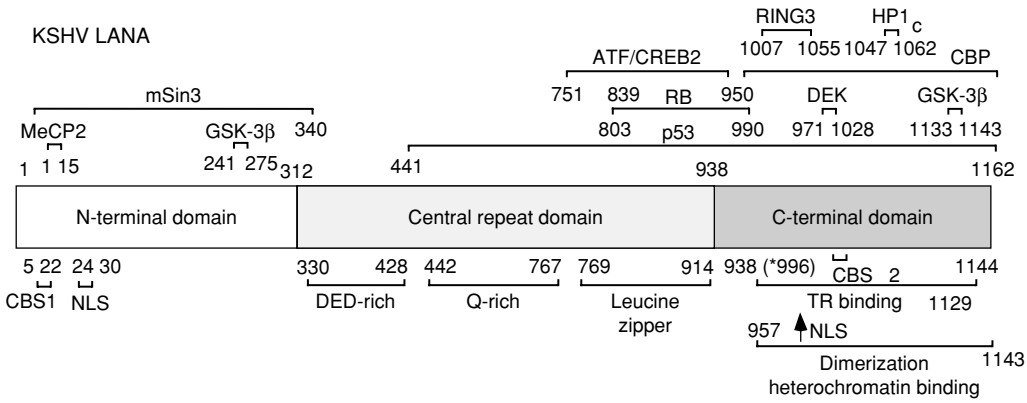

Fig. 24.8. Functional domains of LANA. Amino acid residues are indicated for the boundaries of protein domains for DNA binding, dimerization, chromosome binding sites (CBS), nuclear localization (NLS), or protein-protein interactions (as indicated). LANA amino acid numbering is based on the sequence published by Russo *et al.*, 1996. (* 996 denotes the N-terminus of the core DNA binding domain mapped by Komatsu *et al.*, 2004).

Further evidence that KS lesions harbor episomal DNA comes from the finding that KS spindle cells upon explantation lose viral genomes after only few passages in culture (Dictor *et al.*, 1996). Telomerase-immortalized microvascular endothelial cells can be infected with PEL-derived cell-free virions but lose the viral genome upon cultivation; similar results have recently been shown for a variety of cell lines. These data suggest that the establishment of stable latency at least in the context of *de novo* infection in vitro occurs with low frequency (Lagunoff *et al.*, 2002; Grundhoff and Ganem, 2004). In contrast to cultured cells from KS lesions, PEL cells cultivated in vitro, efficiently maintain KSHV genomes over a long time at a stable copy number (Renne *et al.*, 1996b; Cannon *et al.*, 2000; Cesarman *et al.*, 1995).

Properties of the latency-associated nuclear antigen (LANA)

The first evidence of a latency-associated gene product came from the fact that sera from KS patients contain antibodies that stain PEL cells in a specific nuclear speckled pattern by immunofluourescence assay. This then "unknown" antigen was termed LANA for latency-associated nuclear antigen (Gao *et al.*, 1996; Kedes *et al.*, 1996). Using Northern blot analysis and expression cloning it was subsequently shown that LANA is encoded by ORF73 of KSHV (Kedes *et al.*, 1997; Kellam *et al.*, 1997; Rainbow *et al.*, 1997). *In situ* hybridization studies revealed that nearly all cells in KS lesions express ORF73 mRNA (Dittmer *et al.*, 1998; Talbot *et al.*, 1999) and LANA protein is consistently detected by immunohistochemistry in all malignancies associated with KSHV (Rainbow *et al.*, 1997; Dupin *et al.*, 1999). The ORF73 gene is located in a cluster of four latency-associated genes and is expressed from a poly-cistronic singly spliced mRNA of 5.7 kb that also encodes ORF72, a viral cyclinD homologue, and ORF71, a v-Flip protein (Dittmer *et al.*, 1998; Talbot *et al.*, 1999). Furthermore, this latency-associated region of KSHV contains the Kaposin gene (Zhong *et al.*, 1996; Sadler *et al.*, 1999). The functions and roles of these additional latency-associated gene products in the biology of KS will be discussed elsewhere in this volume and have recently been subject to a number of excellent review articles (Ganem, 1997; Schulz, 1998; Herndier and Ganem, 2001; Boshoff and Weiss, 2002). Adding to the complexity of this region, a cluster of microRNAs, that may modulate host cellular and/or viral gene expression, was recently identified between ORF71 and the Kaposin gene (Cai *et al.*, 2005; Pfeffer *et al.*, 2005; Samols *et al.*, 2005).

Modular domain structure of LANA

LANA can be divided into three major domains: a proline- and serine-rich N-terminal region (312 aa), a central region variable in length between viral isolates (Gao *et al.*, 1999) that is composed of several acidic repeats including a leucine zipper motif, and a conserved C-terminal domain, containing both a proline-rich region and a region rich in charged and hydrophobic amino acids (Fig. 24.8). A comparison of LANA proteins across the rhadinovirus family revealed significant sequence homologies only within the C-terminal domain. While there is some limited

homology within the N-termini, which differ dramatically in size between proteins, there is no conservation for the central domain – indeed this domain is often missing or reduced to a small number of acidic residues (Russo *et al.*, 1996; Grundhoff and Ganem 2003; Alexander *et al.*, 2000; Searles *et al.*, 1999). Although there is only limited amino acid sequence homology between LANA and EBNA1, computer-assisted folding predictions for the LANA C-terminus revealed structural relatedness to the DNA binding domains of EBNA1 and the papillomavirus E2 protein whose structures have been solved (Bochkarev *et al.*, 1996; Grossman and Laimins, 1996; Grundhoff and Ganem, 2003).

Transcriptional regulation and signaling properties of LANA

Two hybrid screens have identified LANA interactions with transcriptional coactivators RING 3 and ATF/CREB (Platt *et al.*, 1999; Lim *et al.*, 2000) and co-repressor mSin3 (Krithivas *et al.*, 2000). Gene expression profiling and transient transcription assays have identified cellular and viral genes that are modulated by LANA (Krithivas *et al.*, 2000; Hyun *et al.*, 2001; Knight *et al.*, 2001; Renne *et al.*, 2001; An *et al.*, 2005). LANA, like EBNA1, transcriptionally activates its own synthesis in part by binding to a low affinity site in its promoter LANAp (Jeong *et al.*, 2001; Renne *et al.*, 2001). On the other hand, Gal4/LANA fusion proteins can repress expression from a Gal4 binding site-containing reporter (Krithivas *et al.*, 2000; Schwam *et al.*, 2000). These results indicate that LANA is a complex transcription regulatory protein that can both activate and repress transcription, by direct DNA binding or indirectly through protein interactions, perhaps also depending on cellular and chromosomal environment. More recently, LANA has also been implicated in early steps that lead to the establishment of latency after *de novo* infection. First, Krishnan *et al.* analyzed viral transcription pattern at very early time points post entry and showed that the earliest gene detectable was ORF50, the viral transactivator which regulates the switch from latent to lytic replication (Krishnan *et al.*, 2004). Next, the expression of LANA was detectable and there was an inverse relation between LANA expression and ORF50 expression. Subsequently, it was demonstrated that LANA inhibits ORF50-dependent transactivation (Lan *et al.*, 2004) and furthermore this inhibitory effect appears to be disrupted by the acetylation of LANA (Lu *et al.*, 2006). These data strongly suggest that LANA expression levels determine the outcome of *de novo* infection. This novel aspect of LANA-dependent regulation of latency and other signaling events that occur early after virus entry are discussed in Chapter 23 of this volume.

LANA also has profound effects on cellular growth regulation and cell cycle control. LANA interacts with p53, and down-regulates p53-dependent transcription (Friborg *et al.*, 2000). LANA was also shown to bind the retinoblastoma tumor suppressor (pRB), and modulates E2F-dependent transcription of cell cycle control genes. Furthermore, ectopic LANA expression can transform RF cells in combination with H-ras (Radkov *et al.*, 2000). Recently, LANA has been shown to promote S-phase entry by targeting the APC/wnt/β-catenin signaling pathway through inactivation with GSK3-beta in primary effusion lymphomas. This pathway is altered in many human malignancies (Fujimuro and Hayward, 2003; Fujimuro *et al.*, 2003). Consistent with these findings, LANA prolongs the passage number of primary human umbilical vein endothelial cells (HUVEC) when introduced by retroviral transduction (Watanabe *et al.*, 2003). However, no experimental system could demonstrate that LANA alone can transform primary cells. In summary, LANA targets key signaling pathways including cell cycle, apoptosis, and chromatin re-modeling and it is, therefore, intriguing to speculate that LANA may contribute to immortalization and/or transformation.

Latent DNA replication and segregation

Ballestas *et al.* first demonstrated through long-term maintenance assays that LANA plays a role in episomal maintenance. Cosmid clones spanning the entire KSHV genome were assayed for their ability to establish plasmid maintenance in BJAB cells in a LANA-dependent manner. Using a selectable marker, resistant cell clones were analyzed for the presence of episomal DNA by Gardella gel analysis. In these assays, a cosmid containing the left end of the genome including terminal repeat (TR) sequences was maintained only in LANA expressing cells. These data unambiguously identified LANA as the only viral protein required in trans for episomal long-term maintenance (Ballestas *et al.*, 1999); these data demonstrated that with respect to its role in episomal maintenance, KSHV LANA is a functional homologue of the EBV origin binding protein EBNA1.

Mechanism of episomal segregation and chromosome tethering

Confocal microscopy demonstrated colocalization of LANA with both viral episomes and mitotic chromosomes suggesting that LANA, like EBNA1, contributes to

long-term maintenance by tethering viral genomes to chromosomes (Ballestas *et al.*, 1999). The association of LANA with heterochromatin during interphase, and mitotic chromosomes during mitosis, has since been demonstrated by several laboratories, and data from Cotter et al demonstrating LANA/Histone H1 interaction first suggested protein-protein interaction as the underlying mechanism for chromosome tethering (Cotter and Robertson, 1999; Szekely *et al.*, 1999; Schwam *et al.*, 2000; Viejo-Borbolla *et al.*, 2003). By preparing a panel of LANA/GFP fusion proteins, Piolot *et al.* identified an 18 amino acid long chromosome binding site (CBS) within the N-terminus of LANA (aa 5 to 22) that confers chromatin association to GFP (Piolot *et al.*, 2001). Most recently, Barbera *et al.* reported a detailed analysis of the N-terminal CBS and provided biochemical and elegant genetic evidence that LANA tethers episomes to mitotic chromosomes through direct interactions with core histones H2A/B. The N-terminal chromosome attachment domain was found to be both critical and sufficient for the histone interaction, which was confirmed further by structural analysis and modeling (Barbera *et al.*, 2006).

Krithivas *et al.* further proved that LANA attaches to chromatin via protein-protein interactions by identifying two chromatin-associated cellular proteins that interact with LANA by two-hybrid screen and immunoprecipitation. MeCP2 is a methyl CpG binding protein and interacts with LANA through the N-terminal chromosome binding domain, referred to as CBS1. The second protein, DEK, interacts with CBS2 located in the C-terminus of LANA (aa 971–1028) (Fig. 24.8). Convincing evidence that two LANA domains are mediating chromosome attachment, binding to MeCP2 and DEK respectively, came from the observation that LANA does not bind to chromosomes in murine cells which are negative for both proteins. However, transfection of MeCP2 and/or DEK encoding cDNAs restored LANA/chromatin co-localization in mouse cells (Krithivas *et al.*, 2002). Although LANA interactions with MeCP2 and/or DEK are highly specific, it is important to note that for episomal maintenance the association of viral episomes and chromatin is sufficient. This was demonstrated by Shinohara et al who replaced the N-terminus of LANA, containing CBS1, with histone H1. The resulting fusion protein was able to co-localize with mitotic chromosomes and, moreover, as shown for a similar EBNA-1/HMG-Y fusion protein, functions in long-term maintenance assays (Hung *et al.*, 2001; Shinohara *et al.*, 2002; Sears *et al.*, 2003). Together, these data strongly suggest that LANA contributes to episomal long-term maintenance by tethering the viral genomes to chromatin (Fig. 24.1).

Mapping LANA's DNA binding motif and DNA binding domain

The long unique region (LUR) of KSHV is flanked on both sides by about 40 copies of terminal repeats which are believed to be important for circularization of the linear virion DNA after viral entry. Each TR unit of KSHV is 801 bp and 89% GC-rich (Lagunoff and Ganem, 1997). Scanning of the 140 kbp LUR did not reveal any repeats or sequence elements reminiscent of homology to the *OriP* of EBV. Instead, Cotter *et al.* provided the first evidence that LANA binds to the TR sequences of the viral genome. Radio-labeled cosmid clones harboring sequences from both ends of the genome were immunoprecipitated with LANA specific antibodies after DNA was incubated with lysates from LANA expressing cells (Cotter and Robertson, 1999).

Direct evidence that LANA could bind to TR DNA was generated with in vitro translated (Ballestas, 2001) or vaccinia virus expressed LANA proteins (Garber *et al.*, 2001, 2002). Electrophoresis mobility shift assays (EMSA) identified a 18 bp long GC rich sequence motif within TR that is bound by LANA (Ballestas, 2001; Garber *et al.*, 2001). Weaker binding sites within TR and one site outside of TR at the left end of the genome have been described but no further functional data for these sites have been reported (Cotter *et al.*, 2001).

Mutational analysis in combination with EMSA mapped the DNA binding domain to the C-terminus of LANA (residues 938 to 1144) (Cotter *et al.*, 2001; Garber *et al.*, 2001). Recently, additional studies have suggested a core DNA binding domain to a 143 amino acid long peptide that encompass residues 996 to 1129 (Komatsu *et al.*, 2004) (Fig. 24.8).

Identification of a DS-like element within TR

After identifying the DNA binding domain within the LANA C-terminus, quantitative EMSA in combination with DNA footprinting revealed that LANA binds to two adjacent sites within TR. When increasing amounts of purified LANA-C protein were incubated with a radio-labeled probe, a second complex with slower mobility was observed at high protein concentrations and its intensity further increased while the first complex seemed unchanged. In vitro DNaseI footprinting assays verified the presence of a second binding site. A re-examination of the TR sequence revealed that the 17 bp imperfect palindrome of LANA binding site 1 (LBS1) is separated by a 5 bp spacer from a second site (LBS2) with only three nucleotide changes, compared to LBS1. Mutation analysis revealed a cooperative binding mechanism by which the high affinity LBS1 permits

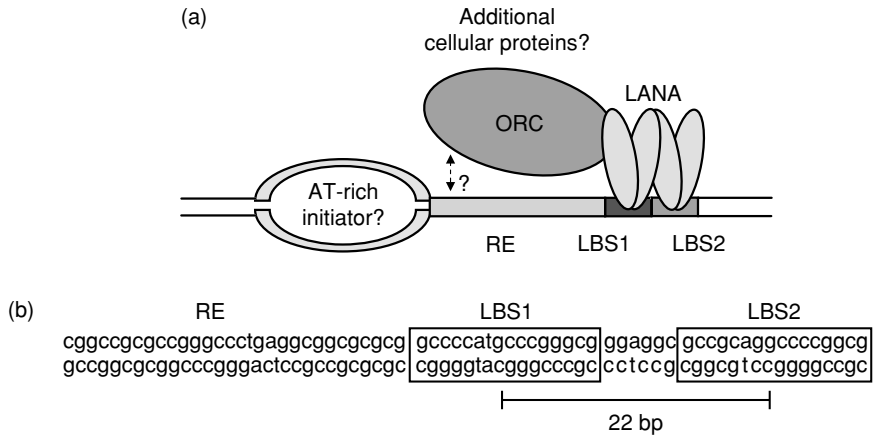

Fig. 24.9. Functional elements of KSHV origin within TR. (a) Model of latent KSHV ori. LBS1/2 and the RE element. LANA binds as dimmers to both sites and the RE element is required for function. ORC LANA and/or TR interaction have been shown both in vitro and in vivo. The AT-rich region may function as initiator element. (b) High (LBS1) and low affinity site (LBS2) are spaced by 22 bps. While this spacing is conserved to DS element of EBV *OriP* there is no sequence homology in binding sites.

loading of LANA onto LBS2 (the Kd for LBS1 was determined to be 1.4 nM) (Garber *et al.*, 2002) (Fig. 24.9).

The presence of both a high and low affinity site and the spacing of 22 bp from center to center within the TR of KSHV is reminiscent of the dyad symmetry element in the EBV *OriP* (21 bp spacing) which is bound by EBNA1 in a cooperative manner. Based on these data and the observation by Schwam et al that the C-terminal DNA binding domain of LANA forms dimers in solution (Schwam *et al.*, 2000), a model is proposed in which two LANA dimers bind to LBS1 and LBS2 in a cooperative fashion, comparable in stoichometry and affinity to EBNA1 binding to *OriP* (Yates *et al.*, 2000).

The TR functions as LANA-dependent plasmid origin

Long-term maintenance of viral DNA in latently infected cells can be divided into two different steps. First, viral DNA needs to be replicated by the cellular replication machinery; second, viral genomes have to be faithfully segregated during mitosis. Ballestas *et al.* showed that plasmids containing two copies of TR sustain LANA-dependent long-term maintenance in lymphoid cell lines (Ballestas *et al.*, 1999)

Short-term replication assays allow the detection of newly synthesized DNA 48 to 72 hours post transfection by its resistance to DpnI digestion, a restriction enzyme that digests only dam methylated DNA. Using this assay several laboratories tested TR-containing plasmids for their ability to replicate in the presence and absence of LANA in primate cells of epithelial, endothelial, and lymphoid lineage. These data established that LANA, in addition to its role in episome segregation, also supports viral DNA replication during latency and that, at least in the context of plasmid replication, all necessary cis-elements are located within the TR sequences (Hu *et al.*, 2002; Lim *et al.*, 2002; Grundhoff and Ganem, 2003). Furthermore, deletion analysis yielded a TR subfragment of 105 bp that replicates as well as plasmids containing one or two copies of TR in the presence of LANA (Hu *et al.*, 2002). Within a TR element, both LANA binding sites contribute to DNA replication since the replication efficiency of mutant TR sequences is directly proportional to their affinity to LANA (Garber *et al.*, 2002).

Further detailed deletion mapping of the minimal replicator within TR revealed that the origin requires LBS1 and LBS2, reminiscent of a dyad symmetry element, and a 29 to 32 bp long GC-rich element upstream. This novel sequence element was termed replication element (RE) since a TR deletion mutant lacking RE does not replicate in the presence of LANA (Hu and Renne, 2005) (Fig. 24.9).

Trans-requirements for LANA and interaction with cellular ORC proteins

Mutational analysis of LANA revealed that the DNA binding domain is required for DNA replication of TR-containing plasmids (Hu *et al.*, 2002) while removal of the central domain has no effect. The C-terminal DNA binding domain of LANA (215 aa) has residual replication activity (25%), and its activity is greatly increased when fused to the N-terminus. This observation is consistent with a model by which the chromosome binding sites located in the N- and C-terminus of LANA contribute to both DNA replication

and segregation (Piolot *et al.*, 2001; Krithivas *et al.*, 2002; Mattsson *et al.*, 2002).

Lim *et al.* has recently demonstrated that the C-terminus of LANA interacts with ORC 2, 3, 4, and 5 in an ATP dependent manner (Lim *et al.*, 2002). These promising in vitro GST pull-down results have been confirmed by in vivo interaction studies demonstrating ORC2 binding to TR in a LANA-dependent manner (Stedman *et al.*, 2004). Thus, in order to support DNA replication both EBNA1 and LANA bind to DS elements in their respective origin and recruit cellular licensing factors (Figs. 24.6 and 24.9).

Summary

All the reported data strongly suggests that the KSHV origin of latent replication is located within the TR sequences of KSHV – as a result, KSHV has many putative origins of replication. However, no genetic studies on KSHV have been reported that address the role of the TR sequences and LANA in the context of the viral genome. For HVS, a related rhadinovirus, it was shown that both the TR sequences and a functional ORF73 gene are required for the establishment of long-term episomal maintenance (Collins *et al.*, 2002). These results further suggest that rhadinoviruses do not contain an *OriP* element within the LUR.

Considerable progress has been made to analyze KSHV latent DNA replication and segregation in plasmid-based assays – many more studies are required to address the question of where DNA replication initiates within the context of the viral episome in vivo.

EBV and KSHV origins and origin binding proteins: conserved and diverged?

EBNA1 and LANA are functional homologues. Both proteins support latent DNA replication by specifically binding to their respective origins of replication and by recruiting host cellular ORC proteins (Figs. 24.6 and 24.9). In addition, EBNA1 and LANA promote long-term episomal maintenance by tethering viral genomes to chromatin.

There is no sequence homology between EBNA1 and LANA binding sites within the DS element of *OriP* and the TR of KSHV. However, there is significant similarity in the structural organization of the minimal replicator elements. Both proteins bind as dimers in a cooperative manner to two sites that have nearly identical spacing from center to center (21 bp for EBV and 22 bp for KSHV). This conserved binding mechanism induces structural changes on DNA such as bending towards the major groove that may directly contribute to origin activity and the formation of pre-replication complexes. LANA binding to TR bends DNA by 57 degrees and 110 degrees when both LBS1 and LBS2 are occupied (Bashew and Yates, 2001; Wong and Wilson, 2005). While for EBV a minimal replicator consists of only two EBNA1 binding sites, the KSHV origin requires a second GC-rich element outside of the LANA binding sites (Fig. 24.10). Additionally, EBNA1 and LANA can both interact with ORC proteins that are likely to be essential for the initiation of DNA replication. It is also likely that EBNA1 and LANA DNA binding domains are structurally similar, and share features with the papillomavirus E2 replication protein.

To facilitate genome segregation and maintenance, both proteins tether episomal viral DNA to cellular chromosomes through protein–protein interactions via multiple domains that target a variety of cellular proteins. Surprisingly, there is little overlap in the set of cellular interacting proteins that mediate attachments to cellular chromosomes for EBNA1 and LANA. The simplest interpretation, that chromosomal attachment can occur in a non-specific manner, has experimentally been supported by the observation that EBNA1 and LANA fusion proteins with known chromatin binding proteins support genome maintenance.

The genomic organization of the maintenance elements in EBV and KSHV appear to have diverged considerably (Fig. 24.10). The 1.2 kbp *OriP* is located within the unique long region of the EBV genome. *OriP* has evolved into two functionally different sequence elements that each contain multiple EBNA-1 binding sites: (i) the dyad symmetry (DS) element, required for EBNA1-dependent DNA replication and (ii) the family of repeats (FR), which are required for long-term maintenance. All lymphocryptoviruses have some *OriP*-like element with conserved genome positioning, although even among the lymphocryptoviruses there is considerable variation on the number and spacing of EBNA1 binding sites, and the requirement for a DS-like element may not exist in the cynomolgous herpesvirus (Fig. 24.2).

The structure of the origin of latent replication within the TRs of the rhadinovirus family is different in that each TR represents a putative origin that contains two LANA binding sites but no distinguishable elements comparable to DS and FR in *OriP*. The repetitive nature of the TRs may reflect some aspects of the EBV FR element; this notion is supported by the observation that more than one copy of TR is necessary for efficient long-term maintenance while a single TR is sufficient for DNA replication. Notably, the EBNA1 repetitive sites within FR are spaced 14 bp, while the LANA binding sites between TR are spaced ~800 bp. Whether this reflects an inherent difference in their mechanism of plasmid maintenance will be interesting to determine.

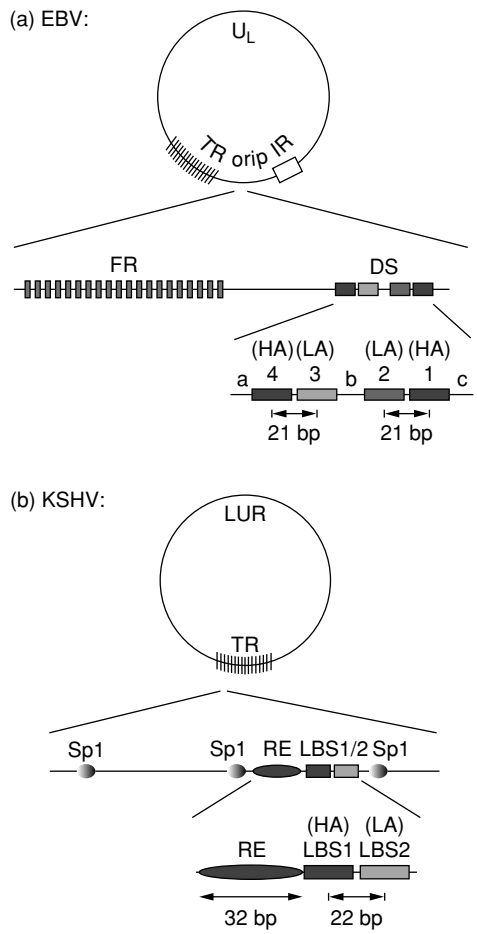

Fig. 24.10. Comparison between genomic structure and positioning of EBV and KSHV. (a) EBV: one *OriP* which contains two functionally different elements that are bound by EBNA1. The DS element contains four EBNA1 binding sites, two sites serve as minimal replicator. To ensure long-term maintenance the FR confer EBNA1-dependent chromatin tethering. (b) KSHV: many putative origins located within TR. Two LANA binding sites resemble DS-like element and together with RE compromise the minimal replicator. The array of TRs may provide tethering function analogous to FR.

The maintenance elements created by repetitive EBNA1 and LANA binding sites are also efficient origins of replication, but these may not be the only replication origins in the large genomes of these viruses. Based on DS deletion studies and single molecule replication data from EBV (Norio *et al.*, 2000; Norio and Schildkraut, 2001), it is likely that both viruses can utilize alternative DNA replication initiation zones in the intact viral genome (Fig. 24.7). The mechanisms governing the utilization of alternative replication initiation sites in EBV or KSHV has not been explored in detail. Nor is it known if the repetitive origins found in the TRs of KSHV function coordinately or selectively to ensure cell cycle restricted genome duplication.

The transcription regulatory properties of EBNA1 and LANA may also be related to their ability to establish replication origins and maintenance elements. Both EBNA1 and LANA may have effects on local chromatin structure which may contribute to the transcription and replication activity at adjacent and distal regions of the genome. The association of LANA with histone modifying complexes and chromatin-associated proteins suggests that much of its activity is directed to altering the chromatin environment.

Some of the growth stimulating activities of LANA, that are not seen in EBNA1, may be attributed to its transcription regulatory and chromatin modulating activities. A similar genomic study of cellular gene expression changes induced by EBNA1 has not been reported, and may reveal unanticipated properties of EBNA1 that are reminiscent of LANA. LANA effects on cell cycle control that can be attributed to the interactions with cellular checkpoint proteins like p53 and Rb, are almost definitely lacking in EBNA1, but perhaps equivalent to the EBNA3 family of interactions. Similarly, LANA's interaction and alteration of the GSK-3β kinase activity and β-catenin signaling pathway may be more equivalent to the activities of EBV transforming proteins encoded by LMP1 and BARTs. This suggests that in addition to their similar but not identical mechanisms for latent DNA replication and genome maintenance, both EBV and KSHV have evolved common strategies to modulate cellular environment in latently infected cells. While in EBV these functions are distributed over an entire family of EBNA proteins, KSHV has evolved a multifunctional protein that has acquired many of the described EBNA activities.

It may also be important to note that both LANA and EBNA1 have a non-essential central linker domain characterized by stretches of copolymeric amino acids. The Gly–Ala repeats in EBNA1 have been shown to have an important function in reducing proteosomal processing and therefore limiting presentation of viral peptide on HLA surface molecules. Recently, a similar immune evasion mechanism was revealed for the highly acidic ED and QP repeats within the central domain of LANA. LANA-GFP fusion proteins containing an ovalbumin epitope are not efficiently presented to cytotoxic T cells. Like the EBNA1 Gly-Ala repeats the LANA central domain conveys reduced antigen presentation in cis (Zaldumbide *et al.*, 2006). Hence, although the sequences of these repeats are not related, this represents yet another example of functional homology between the origin binding proteins of lymphocrypto- and rhadinoviruses.

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25

Reactivation and lytic replication of EBV

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Viral pathogenesis

The lytic form of EBV infection is required for the production of progeny virus, and is thus essential for cell-to-cell spread of the virus, as well as transmission from host to host. Unfortunately, there is currently no cell culture system in vitro that is permissive for efficient primary lytic EBV infection. Although a recent report suggests that allowing the virus to first attach to the surface of primary B cells greatly facilitates EBV infection of epithelial cells in vitro (Shannon-Lowe *et al.*, 2006), even this system of infection still does not result in efficient horizontal spread of virus from cell to cell. Thus, lytic EBV infection in vitro has been studied by reactivating the lytic form of infection from latently infected cell lines using a variety of inducing agents, including phorbol esters, butyrate, calcium ionophores, and B-cell receptor stimulation.

During primary infection, EBV probably initially infects oral epithelial cells in a lytic form, and then subsequently infects B-cells, where the virus usually assumes one of the latent forms of infection. In contrast to alpha and beta herpesviruses, which cause human diseases during the lytic form of viral infection but are essentially innocuous while in the latent form of infection, most illnesses attributable to EBV infection are associated with the latent forms of infection. During primary EBV infection, some individuals, particularly adolescents, develop the syndrome infectious mononucleosis (IM) approximately 1 month after being infected (Cohen, 2000; Jenson, 2000). The EBV-positive B-cells in patients with IM contain primarily the latent form(s) of infection, and the symptoms associated with this illness are attributable to the onset of a vigorous cytotoxic T-cell response against the virally infected B-cells (Cohen, 1999; Jenson, 2000; Andersson *et al.*, 1987; Cohen, 2000). Lytic EBV infection (in either oral epithelial cells or B-cells) presumably precedes the onset of clinical IM, as IM patients have an extremely robust CD8 T-cell response directed against lytic viral protein epitopes (Steven *et al.*, 1997; Hislop *et al.*, 2002). Nevertheless, it has been difficult to document primary lytic EBV infection in patients, presumably due to the long asymptomatic incubation period for IM (Cohen, 1999, 2000).

Following recovery from IM, it is often difficult or impossible to find any lytically-infected cells in immunocompetent individuals (Herrmann *et al.*, 2002), although the preponderance of evidence suggests that reactivated lytic EBV infection most commonly occurs in tonsillar plasma cells as well as in tonsillar B-cells (Laichalk *et al.*, 2005; Pegtel *et al.*, 2004; Niedobitek *et al.*, 1997, 2000; Faulkner *et al.*, 2000). Nevertheless, the only human disease that is unequivocally due to lytic EBV infection, oral hairy leukoplakia (OHL), occurs in epithelial cells on the lateral aspect of the tongue (Lau *et al.*, 1993; Walling *et al.*, 2001, 2003b; Greenspan *et al.*, 1985; Resnick *et al.*, 1990). This hyperproliferative lesion is observed only in highly immunocompromised patients and is easily treated by antiviral agents such as acyclovir that inhibit the lytic form of EBV infection. Interestingly, the lytic (but not latent) form of EBV infection was also recently found in the malignant breast epithelial cells, as well as normal breast cells, in some cases of breast cancer (Huang *et al.*, 2003), suggesting that the breast may also be a site for lytic EBV replication.

The lytic form of EBV infection is clearly required for transmission of the virus from host to host, and thus is an essential aspect of viral pathogenesis. The saliva from immunocompetent hosts often contains infectious EBV (Shu *et al.*, 1992; Lucht *et al.*, 1995; Ikuta *et al.*, 2000; Ling *et al.*, 2003; Walling *et al.*, 2003a), indicating that lytically infected cells in or near the oral cavity must exist, even if they are difficult to detect in the presence of a vigorous cytotoxic T-cell response (Tao *et al.*, 1995). Thus it is not surprising that the great majority (90% or more) of the human

population ultimately becomes infected with this virus. EBV has also been reported to be present in both male and female genital secretions, suggesting that this virus could in some instances be sexually transmitted (Israele *et al.*, 1991; Sixbey *et al.*, 1986).

Activation of lytic EBV infection

Lytic viral gene cascade

EBV lytic genes are expressed in a temporally regulated manner. In many cell lines the two immediate–early genes, BZLF1 and BRLF1, are the first viral genes expressed following lytic induction stimuli, and both BZLF1 and BRLF1 transcription occurs even in the presence of protein synthesis inhibitors (Biggin *et al.*, 1987; Flemington *et al.*, 1991; Takada and Ono, 1989). In some cell lines, such as the Burkitt lymphoma line, Akata, BZLF1 expression may precede BRLF1 expression (Yuan *et al.*, 2006). Once activated, the IE gene products function as transcription factors and together activate transcription of the early viral genes. Early viral genes are defined as genes that are transcribed prior to lytic viral replication (and thus are not inhibited by viral replication inhibitors), but are not transcribed in the presence of protein synthesis inhibitors. Early viral genes encode the viral replication proteins, including the virally encoded DNA polymerase. Following viral replication, the late viral genes are transcribed; late gene transcription is repressed by viral replication inhibitors. Many late genes encode structural proteins that make up the virion particle.

Stimuli that induce lytic EBV infection

In the human host, it is likely that differentiation of B cells into plasma following B-cell receptor stimulation by antigen (Laichalk and Thorley-Lawson, 2005) as well as differentiation in epithelial cells, both activate the lytic form of EBV infection (Tovey *et al.*, 1978; Young *et al.*, 1991). In cell culture systems in vitro, agents such as the phorbol ester, 12-0-tetradecanoyl phorbol-13-acetate (TPA), sodium butyrate, and calcium ionophores are commonly used to induce the lytic form of EBV infection (Faggioni *et al.*, 1986; zur Hausen *et al.*, 1978). Ultimately, what each of these various lytic EBV inducing stimuli shares is the ability to activate transcription of the two EBV IE genes, BZLF1 and BRLF1, from the latent viral genome. Lytic induction stimuli induce BZLF1 and BRLF1 transcription with similar kinetics (Biggin *et al.*, 1987; Flemington *et al.*, 1991; Takada and Ono, 1989), and many stimuli activate both the BZLF1 and BRLF1 promoters in EBV-negative cells (Zalani *et al.*, 1995; Shimizu and Takada, 1993; Feng *et al.*, 2004; Flemington and Speck, 1990d). Thus, other than possibly Akata cells, there is no firm evidence that one IE promoter is more important than the other for mediating lytic induction, and it may be that simultaneous activation of both promoters is required for efficient lytic induction. Once made, the BZLF1 and BRLF1 proteins function as transcription factors which activate their own promoters, as well as one another's promoters (Liu and Speck, 2003; Adamson *et al.*, 2000; Flemington *et al.*, 1991; Ragoczy and Miller, 2001; Sinclair *et al.*, 1991; Speck *et al.*, 1997; Zalani *et al.*, 1996), and thus greatly amplify the inducing effect of the initial lytic stimulus.

Of the various methods used in the lab to induce lytic EBV gene transcription, engagement of the B-cell receptor may be the most physiologic. B-cell receptor activation is accomplished using antibody directed against human IgG or IgM, depending upon which immunoglobulin is produced by the B-cell line. Akata Burkitt lymphoma cells are particularly responsive to this treatment, with up to 50% of cells converting to the lytic form of infection in a synchronous manner. Viral IE gene expression occurs very rapidly (30 minutes or less) after B-cell receptor engagement, followed a few hours later by early gene transcription (Takada and Ono, 1989; Mellinghoff *et al.*, 1991; Flemington *et al.*, 1991). Induction of lytic EBV infection by B-cell receptor engagement requires the activation of calcium-dependent signaling pathways (Chatila *et al.*, 1997; Liu *et al.*, 1997b), as well as the activation of numerous other signaling pathways (Adamson *et al.*, 2000; Bryant and Farrell, 2002; Darr *et al.*, 2001; Iwakiri and Takada, 2004; Mellinghoff *et al.*, 1991). The potential mechanism(s) leading from B-cell receptor engagement to EBV IE gene activation are outlined in Fig. 25.1. The LMP-2A viral latency protein helps to maintain viral latency by preventing the ability of antigen to activate B-cell receptor stimulated signaling pathways (Miller *et al.*, 1994a,b, 1995).

Certain cytokines, particularly TGF-beta, can also induce lytic viral infection in a subset of Burkitt lymphoma lines in vitro, and could potentially reactivate EBV in vivo (Fahmi *et al.*, 2000; Adler *et al.*, 2002). The interaction between CD4 T cells and EBV-infected B cells has also been reported to induce lytic infection (Fu and Cannon, 2000). Finally, there is increasing evidence that severe host cell stress in response to many different toxic stimuli (including chemotherapy and irradiation) can induce lytic EBV infection (Feng *et al.*, 2002a, 2004; Westphal *et al.*, 2000; Roychowdhury *et al.*, 2003).

In oral epithelial cells, lytic EBV infection is normally confined to differentiated cells. In OHL lesions, the EBV genome, and expression of lytic viral proteins, are only found in the more differentiated epithelial cell layers

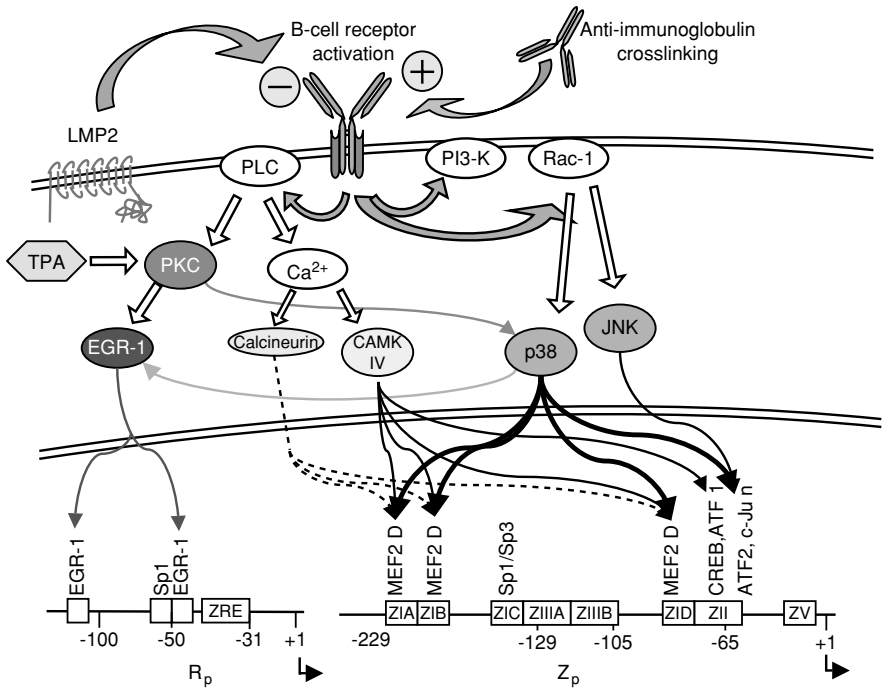

Fig. 25.1. Pathways leading to EBV reactivation in the host cell. Signal transduction pathways that are activated by B-cell receptor (BCR) engagement at the surface of the B-cell, or by phorbol ester (TPA) treatment of cells, are indicated. Promoter motifs in the two EBV IE promoters (Rp and Zp) that are activated by the signaling pathways are shown, as well as the transcription factors that bind to these motifs. Anti-immunoglobulin cross-links and activates the BCR, leading to activation of phosphotidylinositol 3-kinase (PI3K), Ras-family GTPases including Rac1, and phospholipase C gamma-2 (PLC). This is followed by activation of additional downstream pathways as indicated, including protein kinase C (PKC), calcium-dependent factors calcineurin and calcium/calmodulin-dependent kinase type IV (CAMK-IV), and stress MAP kinases (p38 and c-jun N-terminal kinase (JNK)). The EBV LMP-2 protein inhibits activation through the BCR. TPA activates PKC.

(Greenspan *et al.*, 1985; Young *et al.*, 1991). In vitro, differentiation of at least some EBV-positive epithelial lines induces the lytic form of EBV infection (Karimi *et al.*, 1995; Li *et al.*, 1992).

Many of the stimuli used to activate lytic EBV infection in vitro share the ability to activate a variety of signal transduction pathways, including PI3 kinase, p38 kinase, ERK kinase, and protein kinase C, and these kinases have been shown to be essential for induction of lytic EBV transcription by a number of different stimuli (Hayakawa *et al.*, 2003; Mansouri *et al.*, 2003; Fahmi *et al.*, 2000; Fan and Chambers, 2001; Dent *et al.*, 2003; Iwakiri and Takada, 2004; Furukawa *et al.*, 2003; Ionescu *et al.*, 2003; Gao *et al.*, 2001; Satoh *et al.*, 1999; Mellinghoff *et al.*, 1991). In the case of anti-immunoglobulin treatment, activation of calcium-dependent signaling pathways also plays an essential role in lytic viral induction. Two different calcium-dependent proteins activated by B-cell receptor engagement, the calcium/calmodulin-dependent phosphatase, calcineurin, and the calcium/calmodulin-dependent protein kinase type IV/Gr, are required for lytic EBV induction by anti-IgG in Akata cells (Goldfeld *et al.*, 1995; Chatila *et al.*, 1997). Cyclosporin, an immunosuppressive drug that inhibits calcineurin, prevents anti-IgG induction of lytic EBV (Bryant and Farrell, 2002; Goldfeld *et al.*, 1995). The relative importance of each particular signal transduction pathway may vary somewhat depending upon the nature of the stimulus and the cell type.

Organization of the IE gene region of EBV

The organization of the various transcripts and proteins derived from the EBV IE gene region is shown in Fig. 25.2. The immediate–early transcripts that encode BZLF1 and BRLF1 are derived from two different immediate–early promoters, Zp and Rp. The BRLF1 protein is encoded by messages initiating from Rp. These transcripts are bicistronic and could potentially make both the BRLF1 and BZLF1 gene products (Manet *et al.*, 1989). There is some evidence that translation of the BZLF1 protein from the Rp-derived

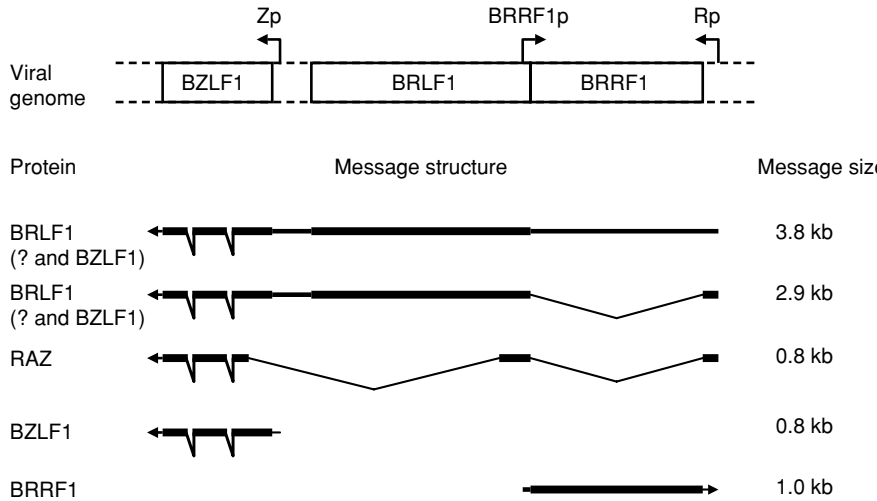

Fig. 25.2. Transcription of the EBV immediate-early gene region. The location of the two immediate-early (IE) genes, BZLF1 and BRLF1, the two IE promoters, Zp and Rp, and the BRRF1 early gene and promoter is shown. Rp directs transcription of a bicistronic message that encodes the BRLF1 and BZLF1 proteins. An alternatively spliced message encodes the RAZ protein, which may function as a negative regulator of BZLF1. The Zp-derived message is the major source of the BZLF1 protein. The BRRF1 promoter directs transcription of the early BRRF1 gene from the opposite DNA strand.

transcripts occurs in vivo, and that the BRLF1 protein is required for this process (Chang *et al.*, 1998; Chang and Liu, 2001). However, the great majority of BZLF1 protein is probably derived from the BZLF1 transcript that initiates from the Zp promoter. A spliced message which contains parts of both BRLF1 and BZLF1 (referred to as RAZ), initiated from Rp, is transcribed later in infection and its gene product may serve as a negative regulator of BZLF1 transcriptional function (Furnari *et al.*, 1994; Manet *et al.*, 1989; Segouffin *et al.*, 1996). The BRRF1 transcript, derived from the BRRF1 promoter and encoded by the opposite strand of the BRLF1 intron (Manet *et al.*, 1989; Segouffin-Cariou *et al.*, 2000), produces an early protein that was recently shown to be a transcriptional activator (Hong *et al.*, 2004) important for efficient lytic EBV gene expression under certain circumstances.

Initial steps in viral reactivation

The promoters driving BZLF1 and BRLF1 transcription, Zp and Rp, are inactive in B-cells containing the latent form of EBV infection (Flemington *et al.*, 1991; Zalani *et al.*, 1992; Biggin *et al.*, 1987; Takada and Ono, 1989; Mellinghoff *et al.*, 1991). Epigenetic modifications such as DNA methylation and histone deacetylation likely contribute to inhibition of IE gene transcription in the context of the intact viral genome (Szyf *et al.*, 1985; Paulson and Speck, 1999; Nonkwelo and Long, 1993; Falk and Ernberg, 1999; Ambinder *et al.*, 1999; Paulson *et al.*, 2002; Bhende *et al.*, 2004; Jenkins *et al.*, 2000; Gruffat *et al.*, 2002b). Nevertheless, even "naked" DNA reporter gene constructs driven by the Zp and Rp promoters are essentially inactive in many EBV-negative B-cell lines (Feng *et al.*, 2004; Kenney *et al.*, 1989b; Sinclair *et al.*, 1991; Zalani *et al.*, 1995), but can be activated by lytic inducing stimuli such as TPA and B-cell receptor stimulation. Thus, the inactivity of the Zp and Rp promoters in unstimulated B cells must reflect the lack of *trans*-acting transcription factors, which positively regulate the two IE promoters, and/or the relative excess of cellular factors which negatively regulate the two IE promoters.

Cellular factors which activate Zp

Cellular transcription factors that positively and negatively regulate the Zp and Rp promoters are shown in Figs. 25.3 and 25.4. Regulation of the Zp promoter has been much more extensively studied than regulation of the Rp promoter. The region of Zp between –233 and +12 contains the *cis*-acting sequences required for Zp activation by lytic-inducing agents. Two types of *cis*-acting motifs, the "ZI" and "ZII" motifs, are critical for activation of Zp by a variety of different lytic-inducing stimuli. The four ZI motifs (ZIA, ZIB, ZIC, and ZID) are AT-rich sequences that have dual roles as both negative, as well as positive, regulators of Zp transcription (Borras *et al.*, 1996; Daibata *et al.*, 1994, Flemington and Speck, 1990a,b; Binne *et al.*, 2002). In the absence of lytic-inducing agents, these elements down-regulate constitutive Zp activity in B cells. However,

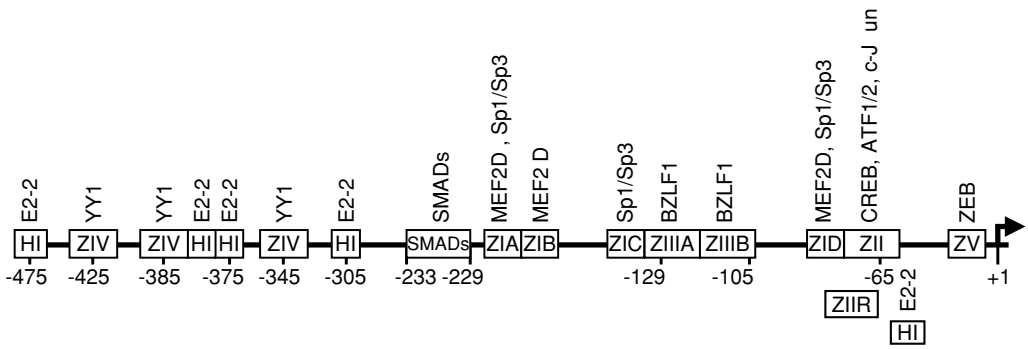

Fig. 25.3. Regulation of the EBV immediate–early BZLF1 promoter. Regulatory motifs in the BZLF1 (Zp) promoter are shown. Cellular and viral proteins known to bind to each motif are indicated.

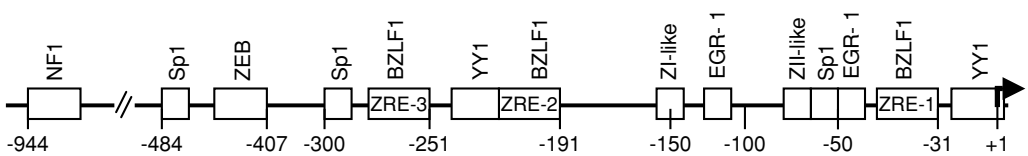

Fig. 25.4. Regulation of the EBV immediate–early BRLF1 promoter. Regulatory motifs in the BRLF1 (Rp) promoter are shown. Cellular and viral proteins known to bind to each motif are indicated.

the ZI elements are also essential for Zp activation by a variety of different inducing stimuli, including TPA, anti-immunoglobulin, calcium ionophores, and chemotherapy (Feng *et al.*, 2004; Binne *et al.*, 2002). Three of the four ZI motifs (ZIA, ZIB, ZID) bind to the transcription factor, MEF2D (Liu *et al.*, 1997b). ZIA, ZIC and ZID also bind weakly to Sp1 and Sp3 (Liu *et al.*, 1997a). This dual role of the ZI motifs as both negative, as well as positive, regulators of Zp transcription may reflect a preferential interaction of MEF2D with histone deacetylating complexes during viral latency (hence acting to inhibit Zp transcription), which is switched to a preferential interaction with acetylating complexes by lytic inducing stimuli (Gruffat *et al.*, 2002b). The phosphorylation status of MEF2D may play an essential role in determining if it represses, versus activates, Zp transcription. Interestingly, calcium/calmodulin-dependent protein kinase type IV/Gr indirectly activates MEF2D by preventing the association between MEF2D and HDAC proteins, whereas MAP kinases directly activate MEF2D by phosphorylating the transcriptional activator domain and enhancing its function (McKinsey *et al.*, 2000). Nevertheless, phosphorylation of other sites in MEF2D may inhibit its function (Li *et al.*, 2001). Cross-linking the B-cell receptor in Akata Burkitt lymphoma cells results in rapid dephosphorylation of the MEF2D in a cyclosporine-sensitive manner, suggesting that calcineurin is involved in dephosphorylating (and perhaps activating) MEF2D (Bryant and Farrell, 2002).

The "ZII" motif is also essential for induction of BZLF1 transcription by most stimuli (Binne *et al.*, 2002; Flamand and Menezes, 1996; Feng *et al.*, 2004; Flemington and Speck, 1990d; Daibata *et al.*, 1994; Chatila *et al.*, 1997). The "ZII" motif is a slightly atypical CREB-responsive element (CRE) that binds CREB, ATF-1, the ATF-2/c-jun heterodimer, and possibly the c-jun/c-fos ("AP1") heterodimer as well (Flamand and Menezes, 1996; Flemington and Speck, 1990d; Liu *et al.*, 1998; Adamson *et al.*, 2000; Wang *et al.*, 1997). The cellular factors which bind to CRE motifs are constitutively expressed in many cell lines, but cannot function as efficient transcriptional activators unless they are phosphorylated over specific residues by certain kinases. The c-jun N-terminal kinase (JNK) is the major activator of c-jun transcriptional function, while the stress Map kinase p38 phosphorylates and activates ATF-2. Many of the stimuli that induce lytic EBV infection in vitro (including B-cell receptor engagement, BRLF1-mediated induction, and certain chemotherapy agents) are known to activate the p38 and JNK kinases, and conversely, inhibitors of p38 and JNK kinases reduce the effectiveness of a number of lytic-inducing stimuli (Adamson *et al.*, 2000; Feng *et al.*, 2002a, 2004). Thus, the activated (phosphorylated) form of an ATF-2/c-jun heterodimer appears to be important for activation of BZLF1 transcription by a number of different stimuli.

Lytic induction may also be mediated through ATF-1 or CREB binding to the ZII motif (Wang *et al.*, 1997), as

the activated (phosphorylated) forms of these transcription factors activate Zp in reporter gene assays. Consistent with a role for ATF- 1 and CREB in at least some cell types, activation of the Zp promoter induced by epithelial cell differentiation is associated with increased binding of ATF-1 and CREB to the Zp CRE motif (Karimi *et al.*, 1995; MacCallum *et al.*, 1999). In addition, engagement of the B-cell receptor results in calcium mobilization and activation of the calcium/calmodulin-dependent protein kinase type IV/Gr, which phosphorylates and activates CREB (Matthews *et al.*, 1994). Both ATF-1 and CREB phosphorylation are observed transiently following anti-IgG treatment of Akata cells (Bryant and Farrell, 2002).

The BZLF1 promoter sequences –233 to –229 bind to Smad3/Smad4, which mediate signaling by the TGF-beta cytokine, and this region of the promoter is required for the TGF-beta activation of Zp that occurs in certain BL lines (Fahmi *et al.*, 2000; Liang *et al.*, 2002). In addition, TGF-beta activation of Zp requires the ZII motif (Liang *et al.*, 2002). C-jun bound to the ZII motif interacts directly with Smad3/4 bound to the Smad binding site in Zp, perhaps explaining the need for both elements (Liang *et al.*, 2002).

BZLF1 autoregulation

There are two ZRE sites in Zp (ZIIIA and ZIIIB), and BZLF1 activates Zp transcription in reporter gene assays (Binne *et al.*, 2002; Flemington and Speck, 1990a). The Zp promoter (in the context of a stable oriP-containing episomal vector) cannot be efficiently activated by anti-IgG signaling in Akata cells when the ZIIIA site of Zp is deleted (Binne *et al.*, 2002). These results suggest that the ability of Z to autoactivate its own transcription may be an essential component for induction of lytic EBV infection. However, it has been suggested that the ZIIIA site in Zp may also bind cellular factors required for induction of BZLF1 transcription by anti-Ig prior to the onset of BZLF1 binding to this site. Since BZLF1 activates TGF-beta transcription (Cayrol and Flemington, 1995), and TGF-beta activates the BZLF1 promoter in at least some cell lines, the TGF-beta pathway may serve as another potential mechanism by which BZLF1 activates its own promoter. Nevertheless, whether BZLF1 actually activates the Zp promoter in the context of the intact genome during viral reactivation remains controversial. A number of published reports suggest that it may not (Binne *et al.*, 2002; Zalani *et al.*, 1996; Le Roux *et al.*, 1996).

Negative regulatory elements in Zp

Negative regulation of BZLF1 transcription may play a critical role in promoting viral latency in B cells. A number of different negative regulatory elements in the BZLF1 promoter have been identified. Of particular importance is the "ZV" motif located near the TATA box that binds to the zinc-finger protein, ZEB (Kraus *et al.*, 2001, 2003). Deletion of this site significantly enhances activation of Zp induced by B-cell receptor engagement in Akata cells (Binne *et al.*, 2002). Binding sites for the cellular YY-1 transcription factor have also been mapped (the "ZIV" elements), and shown to function as negative regulators of BZLF1 transcription (Montalvo *et al.*, 1991, 1995). A *cis*-acting sequence, ZIIR, which overlaps the ZII motif, also reportedly inhibits Zp (Liu *et al.*, 1998), although the cellular factor(s) binding to this repressor have not been identified. The cellular protein, smubp-2, binds to Zp ZI motifs and reportedly functions as a negative regulator, although it is not yet clear whether this negative regulation is mediated by direct binding of smubp-2 to Zp or acts more generally to disrupt formation of a stable TBP-TFIIA-DNA complex (Zhang *et al.*, 1999b). Finally, a series of "H-box" elements, that are similar to E-box motifs and bind to the transcription factor E2–2, have been reported to function as negative regulators of Zp in B-cells, but positive regulators in epithelial cells (Thomas *et al.*, 2003).

Regulation of Rp

Less is known about the regulation of the BRLF1 promoter (Rp) than the BZLF1 promoter. Both the BZLF1 and BRLF1 gene products activate this promoter in EBV-negative cells (Sinclair *et al.*, 1991; Ragoczy and Miller, 2001; Zalani *et al.*, 1992), and the combination of BZLF1 and BRLF1 together is more effective than either protein alone (Liu and Speck, 2003). The Rp promoter, like Zp, is activated by B-cell receptor stimulation, phorbol ester agents, and chemotherapy agents (Zalani *et al.*, 1995; Sinclair *et al.*, 1991; Feng *et al.*, 2004). There are two EGR-1 (Zif-268) binding motifs in Rp, and activation of Rp by TPA and chemotherapy requires these sites in at least some cell types (Zalani *et al.*, 1995; Feng *et al.*, 2004). Phorbol esters, chemotherapy, and B-cell receptor engagement have all been shown to increase the level of cellular EGR-1, suggesting a common mechanism by which these inducing agents might activate BRLF1 transcription. There are also several Sp1 sites in Rp (Zalani *et al.*, 1992), which are required for constitutive promoter activity as well as efficient autoactivation of Rp by its own gene product (Ragoczy and Miller, 2001; Liu and Speck, 2003). A binding site for NF1 has been reported to be a positive regulatory element in HeLa cells but not lymphoid cells (Glaser *et al.*, 1998). There are at least three BZLF1 binding sites (ZREs) in Rp, and BZLF1 activation of Rp is mediated by direct binding of BZLF1 to these ZRE sites (Sinclair *et al.*,

1991; Liu and Speck, 2003; Bhende *et al.*, 2004). Like Zp, the Rp promoter also contains binding sites for the negative regulators, YY-1 and ZEB. The two YY-1 binding motifs function as negative regulators (Zalani *et al.*, 1997) of Rp; the role of the ZEB site, if any, has not yet been defined. Potential ZI-like and ZII-like motifs in Rp exist, but their function has not been studied.

Mechanisms by which TPA and Butyrate activate IE gene transcription

TPA treatment induces lytic EBV gene transcription in some cell lines, and this effect is at least partially mediated through TPA activation of protein kinase C (Gao *et al.*, 2001; Gradoville *et al.*, 2002). Why PKC activation leads to IE gene transcription is not totally clear, but it has been shown to require both the ZI and ZII motifs in Zp (Flemington and Speck, 1990d). In the case of the ZII motif, this may reflect the ability of TPA to activate the stress map kinases (p38 and c-jun N-terminal kinase) in a PKC-dependent manner (Grab *et al.*, 2004; Krappmann *et al.*, 2001), thus leading to phosphorylation of the ATF-2 and c-jun transcription factors which bind to the ZII site. TPA increases the level of EGR-1 in cells (Krappmann *et al.*, 2001), and the Rp EGR-1 binding sites are required for TPA activation of this promoter (Zalani *et al.*, 1995).

Agents that increase histone acetylation by inhibiting histone deacetylase (HDAC) activity, including sodium butyrate, trichostatin A, and valproic acid (Davie, 2003), also induce lytic EBV gene transcription in some cell lines. These agents presumably act by increasing the histone acetylation state of the two viral IE gene promoters (Jenkins *et al.*, 2000). Butyrate-responsive cellular genes (only 2% of all genes), like Zp and Rp, often have Sp1/Sp3 binding sites (Davie, 2003). Sp1 and Sp3 interact with HDAC proteins, as does MEF2D. Thus, in the absence of HDAC inhibitors, Sp1/Sp3 and MEF2D bound to the ZI motifs in Zp may act to inhibit Zp transcription by tethering HDAC complexes to the promoter, whereas in the presence of HDAC inhibitors these transcription factors instead interact with histone acetylases and activate Zp (Gruffat *et al.*, 2002b; Davie, 2003). Unlike the inducing effect of TPA, which requires PKC, the inducing effect of histone deacetylase inhibitors is PKC-independent (Gradoville *et al.*, 2002). HDAC inhibitors alone are insufficient to induce lytic EBV infection in many cell lines (Gradoville *et al.*, 2002), presumably because additional *trans*-acting factors are required to activate BZLF1 or BRLF1 transcription, and the combination of TPA and sodium butyrate is thus required for efficient induction of lytic viral gene expression in many cell lines.

Host cell and viral factors which influence stringency of viral latency

Of note, even in the most susceptible cell lines (generally BL lines), fewer than 50% of cells ever enter the lytic form of infection even when a combination of inducing agents is used. Why a portion of cells always remain in the latent form of viral infection (Gradoville *et al.*, 1990), while others switch to the lytic form of infection, is not currently well understood. In general, we have found that lymphoblastoid cell lines (LCLs) that have been extensively passaged are much more resistant to a variety of different inducing agents than newly derived lines, which often contain a portion of cells in the lytic form of infection even in the absence of inducing agents. In contrast to extensively passaged LCLs, BL lines often retain the ability to respond to one or more lytic-inducing agents.

The tendency of certain cell lines to remain tightly latent even in the face of multiple different inducing agents may be due to either viral and/or cellular factors. Viral factors that promote EBV latency include LMP-2A expression (since LMP-2A inhibits B-cell receptor mediated activation of Zp and Rp), as well as epigenetic modifications of Zp and Rp (including DNA methylation and/or chromatin deacetylation) (Gradoville *et al.*, 1990, 2002; Paulson and Speck, 1999; Nonkwelo and Long, 1993; Szyf *et al.*, 1985). Complete resistance to lytic inducing agents occasionally reflects an integrated viral genome. In some cell lines, such as the Raji BL line, inducing agents cause an abortive lytic infection, with expression of IE and early genes but no lytic viral replication, due to the deletion of one or more essential viral replication genes. In contrast, cell lines containing a defective rearranged form of the EBV genome, dHet, as well as wild-type virus, often have particularly high levels of lytic viral replication (Taylor *et al.*, 1989). This is because the defective rearranged viral genome contains the BZLF1 gene product under the control of the constitutively active EBNA-2 latency promoter, Wp (Countryman *et al.*, 1987; Rooney *et al.*, 1988; Grogan *et al.*, 1987). There is some evidence that similar defective rearranged viral genomes occur during natural infection in humans (Gan *et al.*, 2002; Walling *et al.*, 1992).

Cellular factors contributing to EBV latency include the activated (nuclear) form of NF-KB, which directly interacts with BZLF1 and inhibits its transcriptional function (Morrison and Kenney, 2004, Gutsch *et al.*, 1994). Likewise, the retinoic acid receptor also directly interacts with BZLF1 and inhibits its function (Sista *et al.*, 1993). Nitric oxide also potently down-regulates the lytic form of EBV infection (Gao *et al.*, 1999; Kawanishi, 1995). The cellular level of ZEB, which binds to the ZV motif and inhibits Zp activity

(Krauss *et al.*, 2001, 2003), presumably also influences the stringency of EBV latency.

EBV immediate-early proteins

Introduction

Transcription of the BZLF1 and BRLF1 genes results in expression of the BZLF1 (also known at ZEBRA, Z, EB1 and Zta) and BRLF1 (R, Rta) proteins. Both BZLF1 and BRLF1 are transcription factors, and high-level expression of either BZLF1 or BRLF1 (under the control of a strong heterologous promoter) is sufficient to induce the switch from the latent to lytic form of EBV infection in some latently infected cell lines (Chevallier-Greco *et al.*, 1986; Countryman and Miller, 1985; Rooney *et al.*, 1989; Takada *et al.*, 1986; Zalani *et al.*, 1996; Westphal *et al.*, 1999; Ragoczy *et al.*, 1998). However, in many cell lines, BZLF1 is much more effective than BRLF1 for inducing lytic EBV gene expression, and in some cell lines (such as Raji cells and some lymphoblastoid lines) only BZLF1, and not BRLF1, can disrupt viral latency (Ragoczy and Miller, 1999; Zalani *et al.*, 1996; Hong *et al.*, 2004). Thus, activation of BZLF1 expression (and hence regulation of the Zp promoter) may be relatively more important in vivo for inducing lytic EBV infection than activation of BRLF1 expression. In contrast, only the BRLF1 homologue (and not the BZLF1 homologue) can induce lytic infection in latently infected KSHV cell lines.

BZLF1 transcriptional effects

BZLF1 is a homologue to c-jun and c-fos, and binds as a homodimer to AP-1 like motifs (including the consensus AP-1 site) known as Z-responsive elements (ZREs) (Chang *et al.*, 1990; Packham *et al.*, 1990; Lieberman *et al.*, 1990; Lieberman and Berk, 1990; Farrell *et al.*, 1989; Flemington and Speck, 1990a). BZLF1 transcriptionally activates immediate-early, and early, lytic EBV promoters (Urier *et al.*, 1989; Lieberman *et al.*, 1989; Rooney *et al.*, 1989; Kenney *et al.*, 1989b; Holley-Guthrie *et al.*, 1990; Zetterberg *et al.*, 2002). As shown in Fig. 25.5, the amino-terminus of BZLF1 encodes the transactivator domain (Flemington *et al.*, 1992; Deng *et al.*, 2001), as well as a region required for replication but not transcription (Sarisky *et al.*, 1996). DNA binding is mediated through a domain that is highly homologous to the basic DNA binding domains of c-jun and c-fos (Flemington *et al.*, 1994; Farrell *et al.*, 1989). Homodimerization is mediated through a bZIP domain in the carboxy-terminal portion of the protein (Flemington and Speck, 1990c; Kouzarides *et al.*, 1991). BZLF1 does

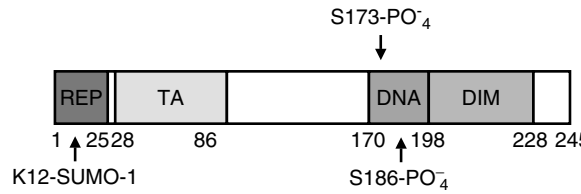

Fig. 25.5. EBV BZLF1 Immediate–early protein. Domains in the BZLF1 protein that mediate dimerization, DNA-binding and transactivation functions are shown, as well as certain phosphorylation and sumo-1 modification sites.

not heterodimerize efficiently with either c-fos or c-jun, but heterodimerizes with another cellular bZIP protein, C/EBP-alpha (Wu *et al.*, 2003), and can activate at least some promoters through C/EBP-alpha binding sites. The crystal structure of BZLF1 was recently published (Petosa *et al.*, 2006) and indicates that the bZIP domain in BZLF1 is somewhat unusual in that the carboxy-terminal region of the protein is also required to form a stable dimer.

BZLF1 activation of early lytic EBV promoters is generally mediated through direct binding of BZLF1 to ZRE motifs within the promoters (Flemington and Speck, 1990a; Urier *et al.*, 1989). BZLF1 may also activate certain cellular promoters through a non-DNA binding mechanism (Flemington *et al.*, 1994). In general, at least two ZRE sites are required for efficient activation of early viral promoters (Carey *et al.*, 1992), and these sites are usually located within a few hundred basepairs of the transcriptional start site. Once bound to DNA, the ability of BZLF1 to interact directly with histone acetylating complexes (including CBP and p300) results in acetylation of chromatin, converting it to a conformation favorable for transcription (Adamson and Kenney, 1999; Chen *et al.*, 2001a; Deng *et al.*, 2003; Zerby *et al.*, 1999). BZLF1 also interacts directly with a number of basic transcription factors, including TFIID and TFIIA (Chi and Carey, 1993; Chi *et al.*, 1995; Lieberman and Berk, 1991, 1994; Lieberman *et al.*, 1997; Mikaelian *et al.*, 1993).

In the context of the intact viral genome, all evidence to date suggests that BZLF1 activation of the BRLF1 IE promoter precedes activation of the early lytic promoters, and that both the BZLF1 and BRLF1 gene products are required for activation of most early lytic genes (Feederle *et al.*, 2000). BZLF1 may bind to the atypical ZRE sites in the BRLF1 promoter in a somewhat different manner (or perhaps conformation) than it binds to the consensus AP-1 site (El-Guindy *et al.*, 2002). This point is perhaps most clearly indicated by the phenotype of a mutant BZLF1 protein in which serine 186 in the basic DNA binding domain is altered to alanine (the residue encoded by the analogous region in c-jun and c-fos). The Z(S186A) mutant cannot bind

efficiently to either of the two ZRE sites in the BRLF1 promoter, although it binds efficiently to the consensus AP-1 site and a variety of ZRE sites within early EBV gene promoters (Adamson and Kenney, 1998; Francis *et al.*, 1997). When transfected into latently infected cells, Z(S186A) cannot induce BRLF1 transcription, and consequently is completely defective for inducing early lytic EBV gene transcription, but its lytic defect is rescued by co-transfection with a BRLF1 expression vector (Francis *et al.*, 1999; Adamson and Kenney, 1998). Serine residue 186 is phosphorylated by PKC in vitro, but whether this phosphorylation actually occurs in vivo remains controversial (El-Guindy *et al.*, 2002; Baumann *et al.*, 1998; Gradoville *et al.*, 2002; Daibata *et al.*, 1992). In lytically-infected B95–8 cells, BZLF1 is phosphorylated at residues Thr 14, Ser167, Ser173 and Ser186, and may be weakly phosphorylated at additional residues (EI-Guindy *et al.*, 2004, 2006). Phosphorylation of BZLF1 residues Ser167 and Ser173 by casein Kinase II, while not required for Z activation of early lytic genes, is required for efficient viral replication and modulates the ability of BRLF1 to regulate late gene transcription (EI-Guindy and Miller, 2004).

BZLF1 activation of methylated ZRE motifs

The EBV genome is highly methylated during the latent form of viral infection, and DNA methylation of promoters generally acts as a potent inhibitor of cellular gene transcription. However, BZLF1 was recently shown to preferentially bind to the methylated vs. unmethylated, forms of two ZRE sites in Rp (Bhende *et al.*, 2004). BZLF1 binding to the ZRE-2 site in Rp, which contains the sequence TGAGCGA, is much enhanced when the cytosine in this motif is methylated, and a previously unrecognized ZRE site in Rp, ZRE-3, which contains the sequence TTCGCGA, can only be bound by BZLF1 in the methylated form. Furthermore, BZLF1 preferentially activates the methylated form of the BRLF1 promoter in reporter-gene assays, and preferentially induces lytic EBV transcription from a methylated versus unmethylated, viral genome (Bhende *et al.*, 2004). Thus, BZLF1 is the first example of a transcription factor that preferentially activates the methylated form of a downstream target gene. This unexpected ability of BZLF1 to activate methylated lytic viral promoters reveals a novel mechanism by which EBV circumvents the inhibitory effects of viral genome methylation.

BZLF1 activation of cellular genes

Not surprisingly, BZLF1 also transcriptionally activates certain cellular genes, some of which may be important for EBV pathogenesis. The cellular genes known to be activated by BZLF1 include TGF-beta (Cayrol and Flemington, 1995), c-fos (Flemington and Speck, 1990b), the tyrosine kinase TKT (Lu *et al.*, 2000), matrix metalloproteinases 1 and 9 (Lu *et al.*, 2003; Yoshizaki *et al.*, 1999), and cellular IL-10 (Mahot *et al.*, 2003). BZLF1 activation of the immunosuppressive cytokines, TGF-beta and IL-10, could potentially dampen the host immune response during the lytic form of virus infection, whereas induction of the matrix metalloproteinases could potentially enhance metastasis of EBV-positive tumors cells expressing BZLF1. In addition, cellular IL-10 is a potent B-cell growth factor, suggesting a mechanism by which lytic EBV gene expression in a small percentage of cells could promote B-cell malignancies in a paracrine manner.

BZLF1 replication function

In addition to its essential role as a transcription factor, BZLF1 also plays a direct role in lytic viral replication. BZLF1 binds directly to a number of ZRE sites in the lytic origin of replication, oriLyt, and this binding is required for oriLyt replication (Fixman *et al.*, 1992, 1995; Hammerschmidt and Sugden, 1988; Schepers *et al.*, 1993a,b). Furthermore, a BZLF1 mutant altered at residues 12/13 is transcriptionally competent, but completely defective for mediating viral replication (Sarisky *et al.*, 1996). BZLF1 also interacts directly with some of the core viral replication proteins (Zhang *et al.*, 1996; Takagi *et al.*, 1991; Gao *et al.*, 1998). Together, these results suggest that BZLF1 acts as an essential oriLyt binding protein during lytic EBV replication, and that this binding may promote formation of the initial replication complex.

The BZLF1-knockout virus is less efficient in promoting lymphoproliferative disease in SCID mice

The phenotype of a BZLF1-deleted EBV has been recently described (Feederle *et al.*, 2000) in 293 cells and primary B cells. As expected, this mutant cannot undergo the lytic form of EBV replication unless the BZLF1 gene product is expressed in trans. In 293 cells infected with the BZLF1-knockout virus, expression of the BZLF1 gene product induces expression of the IE protein, BRLF1, as well as the complete complement of early and late lytic genes. In contrast, expression of the BRLF1 gene product in 293 cells infected with BZLF1-knockout virus does not result in expression of the majority of early or late genes. The BZLF1-knockout virus is not reported to be defective in immortalizing B cells. The phenotype of the BZLF1-knockout virus confirms that both BZLF1 and BRLF1 transcriptional

functions are required for the induction of many (but not all) lytic EBV genes in the context of the intact viral genome.

Surprisingly, however, recent findings suggest that early-passage lymphoblastoid cell lines (LCLs) derived from either BZLF1 -deleted, or BRLF1-deleted, viruses are less efficient than lines derived using wild-type virus in regard to their ability to form lymphoproliferative disease in SCID mice (Hong *et al.*, 2005a,b). LCLs containing the BZLF1-deleted virus secrets less of the two B-cell growth factors, cellular IL-6 and cellular IL-10, and less of the potent angiogenesis factor, VEGF, than LCLs from the same donor containing wild-type EBV. These results suggest that a small number of lytically infected cells may contribute to the growth of some EBV-associated tumors in vivo through the release of paracrine growth factors or angiogenesis factors.

BZLF1 effects on the host cell environment

In addition to its essential roles as a transcription factor and viral replication protein, BZLF1 alters the host cell environment in numerous different ways that presumably act together to enhance the efficiency of lytic viral replication. As the first viral protein expressed during lytic reactivation (and primary lytic infection), BZLF1 is ideally situated to protect the virus from a variety of different host defenses, including cellular apoptosis and the host innate immunity, and to regulate the host cell cycle.

BZLF1 cell cycle effects

There is increasing evidence that herpesviruses usurp the host cell cycle control mechanisms to assure adequate substrates for lytic viral DNA replication. However, the cell cycle effects of BZLF1 appear to be cell-type dependent. BZLF1 produces a profound G1/S block in some cell types, including primary fibroblasts (Rodriguez *et al.*, 1999, 2001a; Cayrol and Flemington, 1995, 1996a,b; Mauser *et al.*, 2002b; Wu *et al.*, 2003). In cell types susceptible to this G1/S block, BZLF1 decreases expression of cyclin A and c-myc (Mauser *et al.*, 2002b; Rodriguez *et al.*, 2001b), and increases p21 expression (Wu *et al.*, 2003; Cayrol and Flemington, 1996b). BZLF1 activates p21 expression through a C/EBP-alpha binding site in the p21 promoter, an effect that involves the direct interaction between BZLF1 and C/EBP-alpha (Wu *et al.*, 2003). In other cell types, such as HeLa cells, BZLF1 induces both a G2 and mitotic block (Mauser *et al.*, 2002a; Cayrol and Flemington, 1996a). The G2 block results from decreased cyclin B, while the mitotic block is associated with a defect in chromosome condensation (Mauser *et al.*, 2002a).

Nevertheless, the cell types in which BZLF1 induces cell cycle blocks are either not normally infected by EBV (fibroblasts), and/or are likely to be deficient in normal cell cycle regulation controls (tumor cells). In sharp contrast to the results in fibroblasts and tumor cell lines, in telomerase-immortalized, as well as primary, keratinocytes, BZLF1 actually enhances expression of a number of S-phase dependent cellular proteins, and increases the level of E2F-1, cyclin E and cyclin A (Mauser *et al.*, 2002b). Likewise, inducible BZLF1 expression in the EBV-immortalized marmoset B-cell line, B95–8, results in enhanced activity of cyclin-dependent kinases, although cellular DNA replication is blocked (Kudoh *et al.*, 2003). Most importantly, agents that inhibit the activity of cyclin-dependent kinases also inhibit lytic EBV gene expression (Kudoh *et al.*, 2004), although it is not currently understood why cyclin-dependent kinases are required for efficient lytic EBV replication. These results suggest that a "pseudo-lateG1/S-phase" environment, in which certain late G1/S-phase restricted cellular proteins are expressed, but cellular DNA does not actually replicate, may be the ideal host cell environment for lytic EBV replication. Inhibition of cellular DNA replication presumably decreases competition between the virus and host cell DNA for limiting substrates involved in DNA replication, while the expression of certain G1/S-phase restricted cellular proteins, such as E2F-1, may be required for viral replication.

BZLF1 effects on p53

Activation of p53 in host cells serves as an important host defense mechanism, since p53 induces cellular apoptosis and hence limits viral replication. Not surprisingly, therefore, many viruses, including herpesviruses, encode proteins that inhibit various aspects of p53 function. The effects of BZLF1 on p53 in the host cell are quite complex. Somewhat paradoxically, in some (but not all) cell types, the presence of BZLF1 results in a rather dramatic increase in the level of total p53, and induces a number of post-translation modifications of p53 (including a series of activating phosphorylations and acetylations) that are usually associated with enhanced p53 transcriptional function (Mauser *et al.*, 2002c). BZLF1 also increases the amount of p53 binding in some cell types (Mauser *et al.*, 2002c). This activation of p53 that occurs in BZLF1-expressing cells may represent an attempt by the host cell to limit EBV replication. Nevertheless, the majority of evidence suggests that BZLF1 quite efficiently inhibits p53 transcriptional function (Zhang *et al.*, 1994; Mauser *et al.*, 2002c). BZLF1 inhibition of p53 may be due in part to the previously observed direct interaction between the BZLF1 and p53 proteins (Zhang *et al.*, 1994). In addition, BZLF1 significantly reduces the level of the basic transcription factor, TATA-binding protein (TBP), in host cells, and restoration

of this protein partially reverses the ability of BZLF1 to inhibit p53 transcriptional function (Mauser *et al.*, 2002c). Finally, as discussed below, BZLF1-mediated dispersion of nuclear PML bodies in host cells may also decrease p53 function, since optimal p53 transcriptional function requires PML bodies. In any event, given that p53 is an important cellular mediator of apoptosis, the ability of BZLF1 to inhibit p53 function likely plays a crucial role in protecting the virus from apoptosis during the earliest timepoints of lytic infection.

BZLF1 dispersion of PML bodies

Promyelocytic leukemia (PML) bodies, also known as nuclear domain 10 (ND-10) bodies, are nuclear structures which contain a number of different cellular proteins, including CREB-binding protein (CBP), Sp100, Rb, Daxx, ISG20, and the small ubiquitin-related modifier, sumo-1 (Bernardi and Pandolfi, 2003; Salomoni and Pandolfi, 2002). Only the PML protein is absolutely essential for formation of PML bodies, and the PML protein must be covalently modified by sumo-1 in order to form these bodies. Although the exact function(s) of PML bodies is somewhat mysterious, the fact that the formation of these bodies is dramatically enhanced by both type I and type II interferons, and that a number of different viruses encode proteins capable of dispersing PML bodies, suggests that these bodies have an antiviral function (Chee *et al.*, 2003; Bernardi and Pandolfi, 2003; Salomoni and Pandolfi, 2002). PML bodies have been proposed to be important for certain types of apoptosis, MHC class I presentation, efficient acetylation of p53, interferon effects, and for the stability and function of an important cellular DNA repair complex, Mre11/Rad50/NBS1 (Bernardi and Pandolfi, 2003; Salomoni and Pandolfi, 2002; Chee *et al.*, 2003). As each of these proposed functions would be expected to reduce viral replication, it is perhaps not surprising that many viruses attempt to inhibit the formation of PML bodies.

High level BZLF1 expression in EBV-negative cells is sufficient to disperse PML bodies, and this effect is correlated with the ability of BZLF1 to inhibit sumo-1 modification of the PML protein (Bell *et al.*, 2000; Adamson and Kenney, 2001). BZLF1 itself is efficiently modified by sumo-1 over lysine 12, and the ability of BZLF1 to inhibit PML protein sumo-1 modification may be at least partially due to competition between BZLF1 and PML for limiting amounts of sumo-1 in the host cell (Adamson and Kenney, 2001). A BZLF1 mutant altered at residues 12 and 13 is transcriptionally competent, but defective in mediating lytic replication (Sarisky *et al.*, 1996). Thus, sumo-1 modification of BZLF1 may be primarily important for its replicative, rather than transcriptional, function.

In the context of the intact virus, lytic EBV infection in cells results in the release of proteins such as Sp100 and Daxx from ND10 bodies, followed by the release of PML (Bell *et al.*, 2000). Lytic viral replication commences after dispersion of the Sp100 and Daxx proteins, but prior to the onset of PML dispersion, and lytic viral replication complexes are often closely associated with dispersed PML protein aggregates (Bell *et al.*, 2000). These findings suggest that reorganized PML complexes may play a role in promoting lytic EBV replication.

BZLF1 effects on the host immune response

BZLF1 plays a key role in attenuating the host immune response to lytic viral infection. PML bodies are required for an efficient antiviral effect of interferon alpha in herpes simplex virus 1 infection (Chee *et al.*, 2003), suggesting that BZLF1-mediated dispersion of PML bodies may protect lytic EBV infection from interferon alpha. BZLF1 also strongly inhibits transcription of the gene encoding an essential component of the interferon gamma receptor, and thereby abrogates interferon gamma signaling in host cells (Morrison *et al.*, 2001). This ability of BZLF1 to inhibit interferon gamma signaling is likely important not only for protecting the virus from the immunostimulatory effects of interferon gamma (including induction of MHC class II and IRF-1), but may also be required for epithelial cell-derived virus to efficiently infect B cells. Virus produced in cells expressing MHC class II, which would normally be induced by gamma interferon in infected epithelial cells if BZLF1 did not prevent this, preferentially infects epithelial cells, whereas virus produced in cells not expressing MHC class II preferentially infects B cells (Borza and Hutt-Fletcher, 2002). In addition to preventing interferon gamma signaling, BZLF1 was recently shown to directly interact with, and inhibit the function of, IRF7 (Hahn *et al.*, 2005). AS IRF7 augments production of type I interferons, the interaction between BZLF1 and IRF7 no doubt helps the virus to attenuate the antiviral effects of interferon alpha and beta in lytically infected cells.

Tumor necrosis factor alpha (TNF-alpha) is another important antiviral cytokine inhibited by BZLF1 expression in host cells. TNF-alpha not only activates expression of a number of important inflammatory genes (through its effects on NF-KB), but also induces cellular apoptosis. As TNF-alpha production is an immediate, and important component of the host immune response to viral infection, not surprisingly many viruses encode proteins that limit the effects of TNF-alpha in the infected host cell. In the case of lytic EBV infection, BZLF1 dramatically inhibits the activity of the promoter for the gene encoding the major

TNF-alpha receptor (TNF-R1) (Morrison *et al.*, 2004). Since TNF-R1 is a fairly short-lived protein, this reduction in TNF-R1 transcription results in dramatically decreased expression of the TNF-R1 protein. Thus, TNF-alpha cannot activate transcription of important downstream target genes such as ICAM-1 in BZLF1-expressing cells, and is also unable to induce cellular apoptosis (Morrison *et al.*, 2004). The exact mechanism(s) by which BZLF1 inhibits transcription of the genes encoding the TNF-R1 and interferon gamma receptors has not yet been defined.

Complex interactions between BZLF1 and the NF-KB transcription factor are also involved in inhibiting expression of many different NF-KB dependent cellular genes involved in the host immune response. BZLF1 interacts directly with the p65 component of NF-KB, and this interaction inhibits the transcriptional function of BZLF1 (Gutsch *et al.*, 1994; Hong *et al.*, 1997). However, recent evidence suggests that BZLF1 also potently inhibits NF-KB-dependent activation of promoters (Keating *et al.*, 2002; Morrison and Kenney, 2004), and decreases NF-KB binding to promoters in the context of the intact cellular genome (Morrison and Kenney, 2004). Thus, the IL-1 cytokine cannot activate NF-KB-responsive cellular genes in the presence of BZLF1, even though the upstream components of the IL-1 signaling pathway appear to be unaffected by BZLF1 (Morrison and Kenney, 2004). Somewhat paradoxically, however, since one of the NF-KB responsive genes inhibited by BZLF1 is I-kappa B (IK-B) (Morrison and Kenney, 2004), and the IK-B protein normally acts to retain NF-KB in the cytoplasm in an inactive form, BZLF1-expressing cells actually have a very high level of nuclear NF-KB (Morrison and Kenney, 2004). This BZLF1-mediated translocation of NF-KB into the nucleus may act to negatively regulate BZLF1 transcriptional function and hence promote viral latency in situations where BZLF1 expression is limiting relative to the amount of nuclear NF-KB.

Finally, BZLF1 also regulates cellular cytokine expression in ways that would be anticipated to protect the virus. As discussed previously, BZLF1 stimulates expression of both TGF-beta, and IL-10 (Cayrol and Flemington, 1995). Both TGF-beta and IL-10 have immunomodulatory effects that would be expected to attenuate the cytotoxic T cell response directed against the virus. In addition, BZLF1 inhibits expression of MHC class I on cells (Keating *et al.*, 2002; Mahot *et al.*, 2003). This latter effect may be at least partially mediated through BZLF1 effects on NF-KB, although other pathways appear to be involved, as well (Keating *et al.*, 2002). The immunomodulatory effects of BZLF1 are summarized in Fig. 25.6.

The role of BRLF1 in lytic induction

Expression of the EBV immediate–early protein, BRLF1, also induces lytic EBV infection in a subset of latently infected cell lines (Feederle *et al.*, 2000; Ragoczy *et al.*, 1998; Zalani *et al.*, 1996; Westphal *et al.*, 1999). Interestingly, the BRLF1 homologue in KSHV (ORF50) is the major inducer of lytic infection for this virus. Even in cell lines (such as the Raji Burkitt lymphoma line) where BRLF1 expression by itself is not sufficient to disrupt viral latency, it is clear that BRLF1 expression is required (in concert with BZLF1) to activate many early lytic genes, including BMRF1 (Feederle *et al.*, 2000; Ragoczy and Miller, 1999). Consistent with this, the BRLF1 gene product is essential for lytic viral replication (Feederle *et al.*, 2000). Like BZLF1, BRLF1 also binds directly to the EBV oriLyt (Gruffat and Sergeant, 1994; Hammerschmidt and Sugden, 1988). In contrast to the importance of certain oriLyt ZRE sites, the BRLF1 binding sites in oriLyt are not absolutely essential for oriLyt replication, at least in plasmid-based replication assays (Fixman *et al.*, 1992).

The primarily nuclear BRLF1 gene product is a transcriptional activator that contains an amino-terminus DNA binding domain and homodimerization domain (Manet *et al.*, 1991) and a carboxy-terminal transcriptional activation domain (Hardwick *et al.*, 1988, 1992) (Fig. 25.7). The transcriptional activator domain of BRLF1 interacts directly with TBP and TFIIB (Manet *et al.*, 1993). BRLF1 also interacts directly with the histone acetylase, CREB-binding protein (CBP) (Swenson *et al.*, 2001). BRLF1 activates lytic EBV gene promoters through at least two different mechanisms. BRLF1 binds directly to a GC-rich motif (consensus $GGCCN_7GTGGTG$) which is present in the promoters of at least three early EBV genes, SM (formerly called BMLF1), BHRF1, and BMRF1 (Gruffat *et al.*, 1990; Gruffat *et al.*, 1992; Gruffat and Sergeant, 1994; Kenney *et al.*, 1989a; Quinlivan *et al.*, 1993). In the case of the BHRF1 and SM promoters, the BRLF1-binding motifs function as powerful enhancer elements in the presence of the BRLF1 protein (Kenney *et al.*, 1989a; Cox *et al.*, 1990; Chevallier-Greco *et al.*, 1989). In the case of the BMRF1 promoter, BRLF1 by itself induces little activation, but cooperates with BZLF1 to produce efficient activation of this promoter (Holley-Guthrie *et al.*, 1990; Quinlivan *et al.*, 1993).

In contrast, BRLF1 activates its own promoter (Rp) and the BZLF1 promoter (Zp) through mechanisms that do not involve direct DNA binding of BRLF1 to these promoters. BRLF1 stimulation of its own promoter requires the Rp Sp1 motifs (Ragoczy and Miller, 2001; Liu and Speck, 2003), although it is not yet known exactly how (or if) BRLF1 regulates cellular factors binding to the Sp1 motif. BRLF1

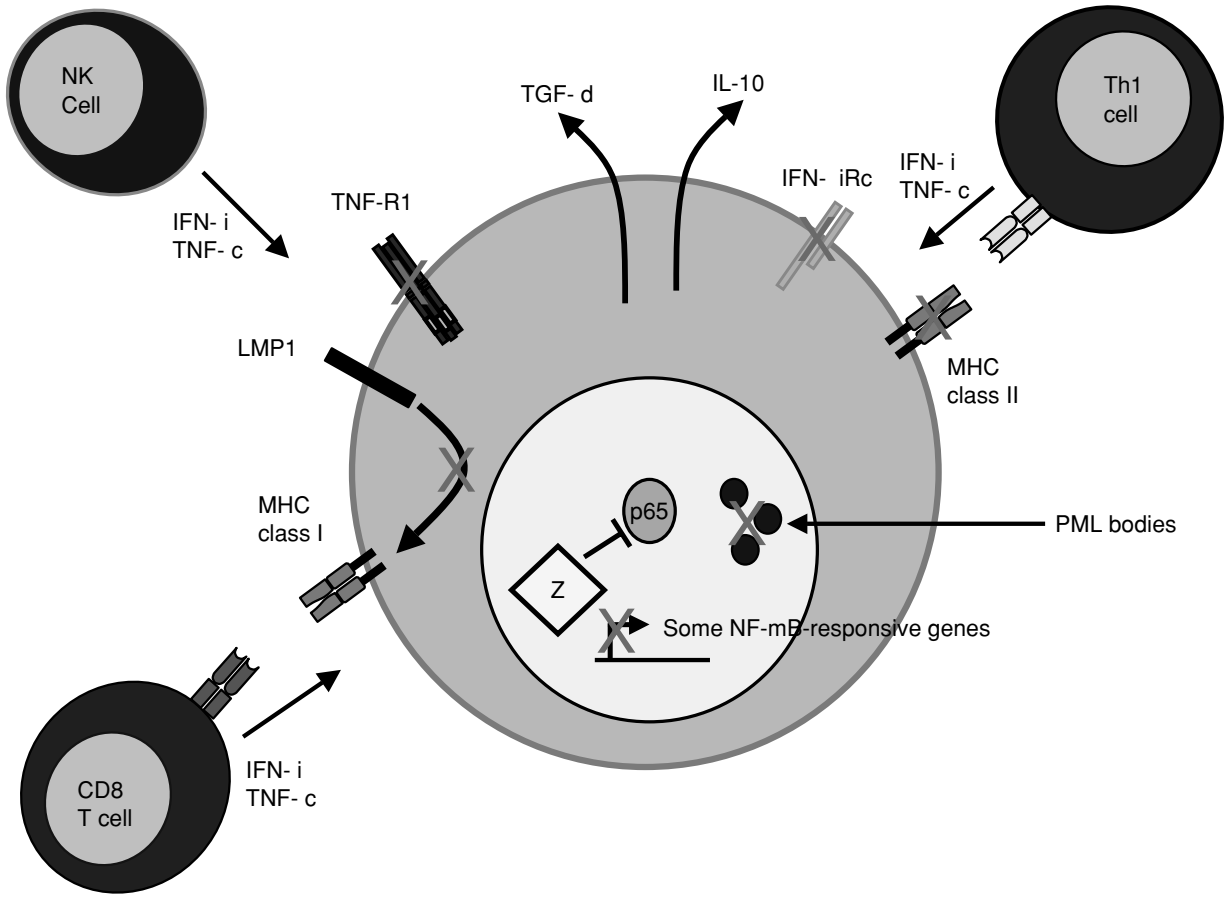

Fig. 25.6. Inhibitory effects of BZLF1 on host immunity. BZLF1 activates transcription of the immunosuppressive cytokines, TGF-beta and IL-10, while decreasing transcription of the receptors for interferon gamma and TNF-alpha. BZLF1 inhibits the transcriptional activity of the p65 component of NF-kappa B and abrogates MHC class I induction by LMP1. BZLF1 also mediates dispersion of PML bodies, which are important for MHC class I expression and response to alpha interferon.

stimulation of the Zp promoter requires the ZII motif, and is likely mediated by BRLF1 activation of the c-jun and ATF-2 transcription factors (Adamson *et al.*, 2000). BRLF1 expression in cells activates the stress Map kinases (p38 and c-jun N-terminal kinase) (Adamson *et al.*, 2000), as well as PI3 kinase (Darr *et al.*, 2001), and inhibition of either p38 stress Map kinase, or PI3 kinase, activity abolishes the ability of BRLF1 to activate BZLF1 transcription, or disrupt viral latency (Adamson *et al.*, 2000; Darr *et al.*, 2001). BRLF1 stimulation of the viral DNA polymerase promoter is also mediated through an indirect mechanism, involving USF and E2F-1 binding motifs (Liu *et al.*, 1996).

The inability of BRLF1 expression by itself to induce fully lytic gene expression in certain cell lines, such as Raji, primarily reflects the inability of BRLF1 to activate BZLF1 transcription in these cell lines (Zalani *et al.*, 1996). In Raji cells, BRLF1 by itself efficiently activates an early promoter (SM promoter) that is directly bound by BRLF1, but cannot activate Zp, or an early viral promoter, BMRF1, which requires the combination of BZLF1 and BRLF1 for activation (Ragoczy and Miller, 1999). Why BRLF1 induces BZLF1 transcription in some latently infected cell lines, but not others, is not currently understood.

BRLF1-knockout virus phenotype

A BRLF1-deleted knockout virus has been made and its phenotype studied in 293 cells as well as primary B cells (Feederle *et al.*, 2000). As is the case for the BZLF1- knockout virus, the BRLF1-knockout virus immortalizes primary B cells with efficiency similar to wild-type virus, but early-passage B cell lines obtained with BRLF1-deleted virus

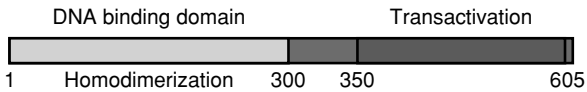

Fig. 25.7. EBV BRLF1 immediate–early protein. Domains in the BRLF1 protein that mediate dimerization, DNA-binding and *trans*-activation functions are shown.

are impaired for producing lymphoproliferative disease in SCID mice (Feederle *et al.*, 2000; Hong *et al.*, 2005a,b). In 293 cells, the BRLF1-knockout virus is unable to enter the lytic form of infection, or express the BZLF1 immediate–early gene, unless the BRLF1 gene product is supplied in trans. These results confirm that the BRLF1 gene product is an important and essential activator of the Zp IE promoter, and that many early viral promoters require both BZLF1 and BRLF1 functions for activation in the context of the intact viral genome. As discussed later, this particular BRLF1-knockout virus was subsequently discovered to be unable to express an early EBV gene product, BRRF1. The phenotype of the "BRLF1-knockout" virus in certain cell types is also partially due to the loss of BRRF1 expression, which cooperates with BRLF1 to activate transcription of BZLF1 (Hong *et al.*, 2004). Nevertheless, it is clear that the BRLF1 gene product is essential for fully lytic EBV gene expression, as well as lytic viral replication, in all cell lines tested to date (Feederle *et al.*, 2000; Hong *et al.*, 2004).

There is emerging evidence that BRLF1 may be able to activate transcription of a subset of late viral genes even in the absence of viral replication. In 293 cells containing the BZLF1-knockout virus, BRLF1 induces expression of a subset of "late" genes, although the virus cannot replicate due to the lack of BZLF1 expression (Feederle *et al.*, 2000). BRLF1 also activates some late genes in Raji cells, in which the viral genome is unable to replicate (Ragoczy and Miller, 1999). The exact mechanism by which BRLF1 activates late genes has not been well defined. Assuming that BRLF1 also activates certain late genes when expressed at a physiologic level in the context of the intact viral genome, the regulation of EBV late gene transcription may be fundamentally different from that of late viral genes in the alpha herpesviruses. The ability of BRLF1 to activate certain viral late genes is modulated by the BZLF1 protein (EI-Guindy and Miller, 2004).

BRLF1 activation of the cellular fatty acid synthase gene

Recent evidence suggests that BRLF1 activation of the cellular gene, fatty acid synthase (FAS), may be an essential component in BRLF1-mediated induction of lytic EBV infection (Li *et al.*, 2004). The FAS enzyme is required for the synthesis of many different lipids, including palmitate, and high-level expression of FAS is normally restricted to fat cells and liver cells. BRLF1 robustly activates FAS gene expression in host cells, and this effect is lost in the presence of p38 kinase inhibitors. Of potential therapeutic interest, agents known to specifically inhibit the FAS enzyme (cerulenin and C75) also inhibit the ability of transfected BRLF1 to induce the lytic form of EBV gene expression, including induction of BZLF1 transcription (Li *et al.*, 2004). In contrast, FAS inhibitors do not affect the ability of transfected BZLF1 (driven by a strong heterologous promoter) to induce lytic EBV gene expression. These results suggest cellular FAS activity is required for BRLF1 activation of the BZLF1 promoter (Zp). Exactly why this is the case remains unknown, but may reflect the fact that palmitoylation of proteins is often required for entry of these proteins into lipid rafts, and the ability of these proteins to initiate signal transduction cascades. Since FAS inhibitors also repress anti-IgG induction of lytic EBV in Akata cells, and the constitutively lytic EBV infection which occurs in AGS (gastric carcinoma) cells (Li *et al.*, 2004), they could potentially be developed as a completely novel approach for inhibiting the earliest aspects of lytic EBV infection in patients.

BRLF1 cell cycle effects

Like BZLF1, BRLF1 also profoundly affects the regulation of the host cell cycle. BRLF1 expression results in an increased number of cells in S-phase in both primary human fibroblasts and HeLa cells, and this effect is associated with dramatically increased E2F-1 expression (Swenson *et al.*, 1999). In addition, since BRLF1 activates the promoter of the viral DNA polymerase gene through an E2F-1 site (rather than a direct binding mechanism) (Liu *et al.*, 1996), the ability of BRLF1 to increase the level of E2F-1 in the host cell may be required for efficient transcription of the viral DNA polymerase (and consequently efficient lytic viral replication). BRLF1 also interacts directly with the tumor suppressor protein, Rb (Zacny *et al.*, 1998). Since Rb plays an essential role in regulating the host cell cycle, the interaction between Rb and BRLF1 may be a mechanism by which BRLF1 activates cell cycle progression.

Early lytic EBV gene regulation

The expression of the two immediate–early proteins, BZLF1 and BRLF1, allows the subsequent expression of viral early genes. In the context of the intact viral genome, transcriptional activation of many early promoters, such as BMRF1, requires that both the BZLF1 and the BRLF1 gene products be expressed (Feederle *et al.*, 2000; Cox *et al.*, 1990). The most extensively studied early lytic EBV promoter is the BMRF1 gene promoter. This promoter contains two BZLF1 binding motifs, as well as one BRLF1 binding site (Holley-Guthrie *et al.*, 1990; Quinlivan *et al.*, 1993). In reporter gene assays in EBV-negative cells, BZLF1 alone efficiently activates the BMRF1 promoter (an effect which requires that both of the ZRE sites be present), whereas the BRLF1 gene product by itself at most modestly activates the BMRF1 promoter. In some cell types, but not others, the combination of BZLF1 and BRLF1 together is significantly more effective than BZLF1 alone at activating BMRF1 reporter gene plasmids (Holley-Guthrie *et al.*, 1990). In cells containing the latent form of EBV infection, all data to date suggest that the combination of both BZLF1 and BRLF1 is required for significant activation of BMRF1 transcription, regardless of cell type (Adamson and Kenney, 1998; Feederle *et al.*, 2000; Ragoczy and Miller, 1999; Zalani *et al.*, 1996).

Nevertheless, the relative importance of the BZLF1 versus BRLF1 gene products for activation of early gene transcription in the context of the intact viral genome appears to be promoter-specific. The early SM promoter, which contains at least one BZLF1 binding motif, as well as a BRLF1 binding site, can be activated by transfected BRLF1 in a cell line (Raji) where BRLF1cannot induce BZLF1 expression from the endogenous viral genome (Ragoczy and Miller, 1999; Swenson *et al.*, 2001). Thus the SM early promoter can be activated by BRLF1 alone, even in the context of the intact viral genome. Two other early promoters, BHLF1 and BHRF1, which are divergent promoters contained within the lytic EBV origin of replication, oriLyt, have been extensively studied in vitro, but much less so in the context of the intact virus. There are two strong BRLF1 binding sites located between the BHLF1 and BHRF1 promoters in oriLyt (Gruffat and Sergeant, 1994); in reporter gene assays, the BHRF1 promoter appears to be more responsive to BRLF1 than the BHLF1 promoter (Hardwick *et al.*, 1988; Cox *et al.*, 1990). Both the BHLF1 and BHRF1 promoters have two or more BZLF1 sites and are activated by BZLF1 alone in reporter gene assays (Hardwick *et al.*, 1988). The relative importance of BZLF1 vs. BRLF1 for BHLF1 vs. BHRF1 transcriptional activation in the context of the intact virus has not been well studied.

Early lytic EBV gene products

Replication proteins

Many of the early lytic EBV gene products are directly involved in mediating the lytic form of viral replication. The six core viral replication proteins which mediate lytic viral replication include the catalytic component of the viral DNA polymerase (BALF5), the DNA polymerase processivity factor (BMRF1), the single-stranded DNA binding protein (BALF2), helicase (BBLF4), primase (BSLF1), and primase-associated protein (BBLF2/3)(Fixman *et al.*, 1992). In addition, some early EBV genes encode enzymes involved in deoxynucleotide metabolism, including the viral thymidine kinase gene (BXLF1) (Littler *et al.*, 1986), the viral dUTPase gene (Fleischmann *et al.*, 2002) and viral ribonucleotide reductase (BORF2 and BARF1). From the results of studies performed on the analogous genes in HSV-1, it is likely that these early EBV gene products are required for efficient production of nucleotide substrates in non-replicating cells. In addition, the EBV-encoded thymidine kinase (TK) function may be required for the therapeutic effect of the antiviral drug, acyclovir, as the EBV TK is a deoxypyrimidine kinase that phosphorylates a broad range of nucleoside analogues (such as acyclovir) not recognized efficiently by the cellular TK (Littler and Arrand, 1988).

Transcription factors

There are at least two viral transcription factors encoded by early lytic EBV genes. Interestingly, the BMRF1 gene product (Cho *et al.*, 1985) functions not only as the viral DNA polymerase-processivity factor (Tsurumi *et al.*, 1993, 1994), but also as a transcription factor (Zhang *et al.*, 1997, 1999a). BMRF1 activates transcription of the oriLyt promoter, BHLF1, and the BMRF1-responsive region of this promoter is contained within a GC-rich domain (the downstream essential domain) that is also essential in *cis* for oriLyt replication (Zhang *et al.*, 1997). The exact mechanism by which BMRF1 activates oriLyt transcription is not yet known. The transcriptional function of BMRF1 requires its carboxy-terminal domain (Zhang *et al.*, 1999a), which is not required for DNA polymerase-processivity function in vitro (Kiehl and Dorsky, 1991; 1995).

Another early lytic viral transcription factor is the BRRF1 gene product (Hong *et al.*, 2004). The BRRF1 gene is encoded by the opposite strand of the first intron of the BRLF1 immediate–early gene (Segouffin-Cariou *et al.*, 2000). BRRF1 enhances the transcriptional function of c-jun, and activates the BZLF1 immediate–early promoter

through the CRE ("ZII") motif (Hong *et al.*, 2004). BRRF1 activation of c-jun transcriptional function is associated with increased c-jun phosphorylation, although the precise mechanism for this effect is not yet known. Although BRRF1 expression alone is not sufficient to activate BZLF1 transcription from the latent viral genome, BRRF1 function is required for efficient BRLF1-mediated activation of Zp in some latently infected cell lines (Hong *et al.*, 2004). A knockout virus originally thought to specifically delete the BRLF1 gene product (Feederle *et al.*, 2000) was subsequently shown to be defective in BRRF1 transcription, and rescue of the fully lytic phenotype of this virus requires both the BRLF1 and BRRF1 gene products in trans (Hong *et al.*, 2004). KSHV contains a gene that is homologous to BRRF1 in the analogous position of the viral genome and has a similar function (Gonzalez *et al.*, 2005).

SM: a protein that regulates RNA transport and stability

In contrast to the latent and IE genes of EBV, the early and late genes often contain no introns. RNA derived from intronless genes is often unstable in cells. The early lytic gene product, SM (previously known as BMLF1) (Cook *et al.*, 1994), is an RNA-binding protein which plays an important role in increasing the stability of intronless lytic viral transcripts, as well as promoting the transport of such messages from the nucleus to the cytoplasm (Kenney *et al.*, 1989c; Gruffat *et al.*, 2002a; Hiriart *et al.*, 2003a,b; Boyle *et al.*, 1999, 2002; Buisson *et al.*, 1989, 1999; Chen *et al.*, 2001b; Farjot *et al.*, 2000; Ruvolo *et al.*, 1998, 2001, 2004; Semmes *et al.*, 1998). The SM protein thus acts to create a cellular environment in which viral lytic (intronless) messages are preferentially expressed over cellular (intron-containing) messages. Recent studies of an SM-knockout virus confirm that the SM gene product is essential for efficient viral replication and virion production (Gruffat *et al.*, 2002a). Another recently described function of the SM protein is its ability to inhibit PKR activation (Poppers *et al.*, 2003), which appears to be distinct from its effect on RNA. PKR inhibition by SM would presumably allow the virus to escape the inhibitory effect of PKR activation on protein translation. In contrast, SM actually enhances the expression of STAT1 (Ruvolo *et al.*, 2003); it is as yet unclear how this activation benefits the virus.

Proteins that inhibit cellular apoptosis and immune evasion

EBV also encodes an early lytic gene product, BHRF1, which inhibits cellular apoptosis. BHRF1 is a homologue of the cellular anti-apoptosis gene, BCL-2, and like BCL-2 inhibits apoptosis in response to a number of different stimuli (Henderson *et al.*, 1993; Tarodi *et al.*, 1994). The ability of BHRF1 to prevent cellular apoptosis very likely increases the efficiency of lytic EBV viral replication by preventing the death of the host cell prior to the completion of replication.

The BARF1 early gene encodes a soluble receptor for colony-stimulating factor 1 (Strockbine *et al.*, 1998). BARF1 inhibits the differentiating and proliferative effects of this cytokine on monocytes/macrophages (Strockbine *et al.*, 1998), and decreases alpha-interferon secretion from monocytes/macrophages (Cohen and Lekstrom, 1999). Thus, BARF1 may be important for protecting the virus from antiviral effects mediated through monocytes and macrophages.

A viral kinase

The BGLF4 early gene encodes a viral kinase (EBV-PK) which is homologous to the UL97 gene product of cytomegalovirus. In addition to autophosphorylation, at least two other EBV proteins, BMRF1 and the EBNA Leader protein, are phosphorylated by the BGLF4 kinase (Kato *et al.*, 2001, 2003; Chen *et al.*, 2000; Gershburg and Pagano, 2002), although the effect of this phosphorylation on BMRF1 and EBNA Leader protein function is not currently defined. In addition, like the CMV (UL97) and HSV (UL13) homologues, BGLF4 also phosphorylates the cellular protein, translation elongation factor 1 delta (Kato *et al.*, 2001). To date the kinase motifs recognized by the BGLF4 and homologous proteins encoded by CMV and HSV appear to be similar to cdc2 kinase motifs (Kawaguchi *et al.*, 2003). Although the exact function of the BGLF4 kinase during lytic EBV replication has not yet been clearly defined, as is the case with the CMV (but not HSV) homologue, BGLF4 expression in cells results in phosphorylation of the antiviral nucleoside analogue, ganciclovir, converting it to the active form (Marschall *et al.*, 2002). Thus, ganciclovir (as well as acyclovir) can be used to inhibit lytic EBV replication.

Viral replication

Lytic EBV replication probably occurs through a rolling-circle mechanism and involves the formation of head-to-tail concatamers of the genome. Lytic EBV replication requires the lytic origin of replication (oriLyt) in cis, and the viral core replication proteins (BALF5, BMRF1, BBLF2, BBLF4, BSLF1, and BBLF2/3) in trans (Fixman *et al.*, 1992,

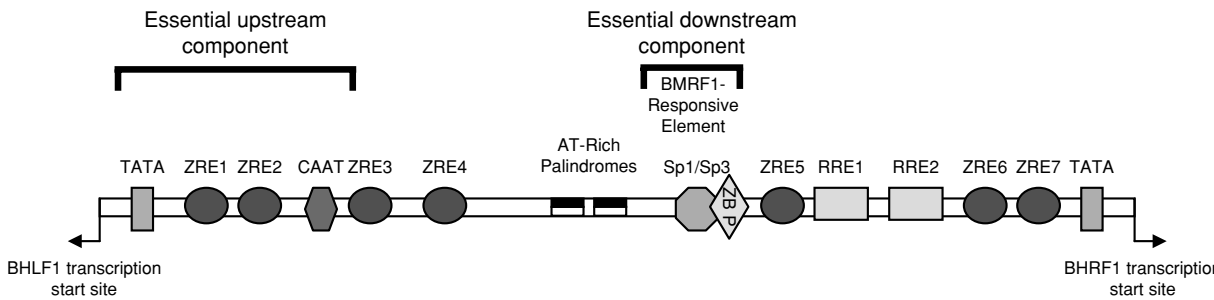

Fig. 25.8. *Cis*-acting elements of oriLyt. OriLyt overlaps the divergent promoters of two early genes, BHLF1 and BHRF1. The locations of BZLF1 (ZRE) and BRLF1 (RRE) binding sites, as well as the binding sites of cellular transcription factors ZBP-89, Sp1, and Sp3, are shown. The localization of two essential elements in oriLyt that are absolutely required for replication in indicated.

1995). Each of these core replication proteins is absolutely essential for the lytic form of replication, and a number of these core replication proteins directly interact with one another, presumably allowing formation of a large replication initiation complex (Daibata and Sairenji, 1993; Fujii *et al.*, 2000; Gao *et al.*, 1998; Liao *et al.*, 2001). In addition, the BZLF1 IE protein is absolutely required for replication, even when the core replication proteins are expressed under strong constitutive heterologous promoters. BZLF1 interacts directly with the core replication proteins, BMRF1 and BBLF4 (Zhang *et al.*, 1996; Liao *et al.*, 2001).

EBV DNA polymerase activity is mediated by the catalytic component of the enzyme (BALF5) in conjunction with the polymerase processivity factor (BMRF1). The catalytic component of the polymerase also has 3′-to 5′ proofreading exonuclease activity (Tsurumi *et al.*, 1993). The EBV polymerase, in contrast to cellular polymerase, is active in high-salt (100 mM ammonium sulfate) (Tsurumi *et al.*, 1993).

There are usually two copies of oriLyt in the EBV genome, although viral strains that contain only one copy of oriLyt (such as B95–8) seem to replicate equally well. The minimal oriLyt contains the divergent promoters of two EBV early genes, BHRF1 and BHLF1, as well as binding sites for the two EBV immediate-early proteins, BZLF1 and BRLF1 (Hammerschmidt and Sugden, 1988) (Fig. 25.8). Two domains of oriLyt are absolutely essential for replication. The "upstream " essential domain contains two binding sites for BZLF1, and BZLF1 binding to these sites is required for replication (Schepers *et al.*, 1993a,b, 1996). The "downstream" essential domain is a GC-rich sequence bound by the Sp1, Sp3 and ZBP-89 cellular transcription factors (Baumann *et al.*, 1999; Gruffat *et al.*, 1995; Schepers *et al.*, 1993b), and this region also mediates BMRF1 transcriptional effects (Zhang *et al.*, 1997). ZBP-89 binding appears to be particularly important for oriLyt replication, and ZBP-89 over-expression in cells enhances replication of an oriLyt containing plasmid (Baumann *et al.*, 1999). In addition, the ability of the downstream essential domain to form a triple helix DNA structure may be required for oriLyt replication (Portes-Sentis *et al.*, 1997).

Once replication is completed, the virus is clipped within the terminal repeat region of the genome into a linear, unit-length genome and then packaged into virion particles. The EBV terminal repeats are sufficient to allow packaging of plasmids in *cis* (Zimmermann and Hammerschmidt, 1995); however, the viral proteins that mediate the cleavage and packaging functions have not been identified.

Late viral gene regulation

Late EBV genes are traditionally defined as genes that are expressed after the onset of viral replication; expression of late genes is inhibited by agents that prevent viral DNA replication. Relatively little is known about the regulation of late EBV promoters. Unlike the early viral promoters, the late promoters do not usually contain BZLF1 or BRLF1 binding sites. Assuming that EBV late viral gene promoters are regulated in a similar manner as the late viral gene promoters in herpes simplex virus, it would be anticipated that EBV late promoters are primarily activated in cis by viral replication, and that this effect requires only a small region of the upstream promoter sequences. However, recent studies using the BZLF1- and BRLF1- knockout viruses have indicated that the requirement for viral replication may not be as absolute for late gene expression in gamma herpesviruses as it appears to be the case for alpha herpesviruses. For example, expression of the BRLF1 immediate-early protein in cells containing the BZLF1-knockout virus induces expression of a subset of "late" genes in the absence of viral replication (Feederle *et al.*, 2000). BRLF1 can also activate some late genes in Raji cells in the absence of viral replication (Ragoczy and Miller, 1999). In reporter gene assays, certain late promoters are

also activated in a replication-independent manner (Serio *et al.*, 1997, 1998).

Late viral proteins

Many late genes encode structural viral proteins, including the nucleocapsid proteins that make up the virion particle. The viral glycoproteins which mediate EBV binding and fusion to the cellular receptor and co-receptor (gp350/220, gp85, gp42, gp25) are also encoded by late genes and are further discussed in Chapter 23. In addition, EBV encodes at least one late viral gene product, vIL-10 (BCLF1), that is likely important for protecting the virus from the host immune response. The vIL-10 gene product is a homologue of cellular IL-10 (Hsu *et al.*, 1990) and shares its ability to potently repress the cytotoxic T-cell response. Therefore, secretion of viral IL-10 from lytically infected cells would be expected to protect the virus from this response (Salek-Ardakani *et al.*, 2002). In addition, as both cellular and viral IL-10 function as B-cell growth factors, the lytically infected pool of EBV-positive B cells could potentially support the growth of the latently infected pool through a paracrine mechanism involving the release of viral IL-10 (Miyazaki *et al.*, 1993; Stuart *et al.*, 1995; Rousset *et al.*, 1992). An EBV mutant virus deleted in the viral IL-10 gene was not found to be defective in B-cell transformation in vitro or lymphoma formation in mice (Swaminathan *et al.*, 1993); however, the lack of an obvious phenotype in this mutant virus may reflect the finding that BZLF1 induces cellular IL-10, and that the functions of cellular and viral IL-10 are redundant. Perhaps not surprisingly, one or more late viral gene products also appear to inhibit apoptosis, suggesting that prevention of cellular apoptosis is important for efficient EBV infection throughout the lytic replication cycle (Inman *et al.*, 2001). A late viral protein which has homology to the anti-apoptotic cellular Bcl-2 protein is encoded by the BALF1 gene, but whether this protein inhibits or activates apoptosis remains controversial (Marshall *et al.*, 1999; Bellows *et al.*, 2002).

Viral assembly and egress

At the end of the lytic replication cycle, the structural proteins involved in initiation of infection must be reassembled into the mature virion. Current models of herpesvirus assembly and egress propose that capsids are first built around a scaffold in the nucleus, that the scaffold is then lost to make room for packaging of the genome, and that the completed nucleocapsids, associated with at least some of the tegument proteins, bud through the inner nuclear membrane into the perinuclear space, acquiring a first envelope in the process. De-enveloped nucleocapsids are then delivered to the cytoplasm by fusion with the outer nuclear membrane or that of the endoplasmic reticulum. The final envelope and additional or different tegument proteins are acquired during rebudding into a cytoplasmic compartment, probably the trans-golgi network, that puts the virus back into a later stage of the exocytic pathway for release by exocytosis (for review see Mettenleiter, 2002). This model has been developed primarily from the study of alphaherpesviruses. However, the probability that EBV follows a similar envelopment, de-envelopment, reenvelopment pathway is supported by observations of the B-958 strain of EBV where higher levels of glycoprotein gB are seen in the nuclear membrane of cells producing virus than in mature enveloped virions and there is a reverse distribution of the major virion glycoprotein gp350/220 (Gong and Kieff, 1990). This is, in turn, dependent on a second soluble nuclear protein, BFLF2 (Gonella *et al.*, 2005; Lake and Hutt-Fletcher, 2004). These two proteins are conserved throughout the herpesvirus family and appear to have similar functions in each. In addition, however, the EBV glycoprotein gB also plays a role in nuclear egress (Herrold *et al.*, 1995). This function is not shared by the gB homologs of the alpha and betaherpesviruses. One possibility is that gB, known to be essential for glycoprotein-mediated cell fusion (Haan *et al.*, 2001), is required for the fusion of the first virus envelope with the outer nuclear membrane. Acquisition of the second and final envelope requires a complex of glycoproteins gN and gM (Lake and Hutt-Fletcher, 2000), but little more is known about the process aside from the provocative finding that loss of the transforming protein LMP1 severely impairs virus release (Ahsan *et al.*, 2005). These final and critical stages in EBV replication remain poorly understood.

Treatment of lytic EBV infection

Lytic EBV infection is inhibited by both acyclovir and ganciclovir (Lin and Machida, 1988; Datta *et al.*, 1980; Lin *et al.*, 1987; Meerbach *et al.*, 1998). The nucleoside analogues, acyclovir and ganciclovir, are phosphorylated to their active forms in lytically infected cells, presumably due to the effects of the two viral kinases, EBV thymidine kinase and BGLF4 (Lin *et al.*, 1986; Moore *et al.*, 2001; Marschall *et al.*, 2002). As acyclovir is less toxic than ganciclovir in patients, acyclovir is the preferred agent for treating the one disease that is definitely due to lytic EBV infection, oral hairy leukoplakia. The antiviral drug foscarnet, which inhibits the viral DNA polymerases of all known herpesviruses and effectively inhibits lytic EBV replication in vitro (Datta and

Hood, 1981), could also theoretically be used to treat lytic EBV infection in patients. However, as foscarnet is quite toxic, and there is no evidence to date that EBV strains resistant to acyclovir or ganciclovir are a clinical problem, foscarnet is not currently used to treat lytic EBV infection in patients. There is no convincing evidence that inhibition of lytic EBV infection in immunocompetent patients with infectious mononucleosis shortens or ameliorates this illness (Torre and Tambini, 1999). Whether acyclovir or ganciclovir treatment is useful in treating early polyclonal EBV-associated lymphoproliferative disease in post-transplant patients is somewhat controversial (Cohen, 2000; Oertel *et al.*, 1999). As lytic EBV gene products are not currently thought to be required for immortalization and growth of B cells in vitro (Feederle *et al.*, 2000), it is not clear that preventing this form of infection in vivo would slow the growth of EBV-immortalized B cells in patients. There is more convincing evidence suggesting that anti-viral prophylaxis reduces the subsequent development of EBV-associated lymphoproliferative disease in transplant recipients (Farmer *et al.*, 2002; Malouf *et al.*, 2002; Green *et al.*, 2001; Fong *et al.*, 2000; McDiarmid *et al.*, 1998; Darenkov *et al.*, 1997). In this case, inhibition of lytic EBV infection presumably reduces the pool of latently infected B cells, thereby decreasing the probability that one or more of these cells eventually becomes malignant.

Lytic induction as a strategy for treating EBV-positive tumors

Finally, the purposeful induction of lytic EBV infection in EBV-positive tumor cells is increasingly being explored as a potential way to selectively kill EBV-infected tumor cells (Gutierrez *et al.*, 1996; Israel and Kenney, 2003; Westphal *et al.*, 1999). Theoretically, EBV-positive tumors containing the latent forms of viral infection could be switched to the lytic form of infection by either inducing expression of the EBV immediate–early genes from the endogenous viral genome in tumors, or by using gene delivery methods to express either of the EBV immediate–early proteins in tumor cells. When adenovirus vectors expressing either the BZLF1 or BRLF1 gene products are injected directly into EBV-positive nasopharyngeal carcinoma tumors grown in nude mice, tumor growth is inhibited, whereas control adenovirus vectors have no effect (Feng *et al.*, 2002b). In addition, certain cytotoxic therapies, including some chemotherapy agents and gamma irradiation, have been found to induce lytic EBV gene transcription in at least a portion of tumor cells (Feng *et al.*, 2002a, 2004; Roychowdhury *et al.*, 2003; Westphal *et al.*, 2000). Lytic induction by cytotoxic agents is mediated through activation of the two EBV immediate-early promoters and requires the PI3 kinase, p38 kinase, and MAP kinase pathways (Feng *et al.*, 2002a, 2004). Specific transcription factor binding sites in both the BZLF1 promoter (ZI and ZII), and the BRLF1 promoter (EGR-1) are also required (Feng *et al.*, 2004). Agents that inhibit histone deacetylases (such as butyrate compounds) have also been shown to enhance the amount of lytic EBV infection in some mouse tumor models (Westphal *et al.*, 2000).

Lytic induction strategies are most effective for inhibiting tumor growth when combined with the antiviral drug, ganciclovir (Mentzer *et al.*, 1998; Feng *et al.*, 2002a, 2004; Faller *et al.*, 2001; Roychowdhury *et al.*, 2003; Westphal *et al.*, 2000). In cells containing the lytic (but not latent) type of EBV infection, virally encoded kinases (BGLF4 and the viral thymidine kinase) are expressed which phosphorylate the nucleoside analogue, ganciclovir, converting it to its active cytotoxic form (Moore *et al.*, 2001). Phosphorylated ganciclovir inhibits not only viral DNA replication, but also inhibits the host cell DNA replication, and is thus cytotoxic. Furthermore, phosphorylated ganciclovir can be transferred into nearby cells that are unable to phosphorylate ganciclovir (i.e., tumor cells with latent EBV infection), and thus induce "bystander" killing. As chemotherapy and irradiation induce lytic infection in only a portion of tumor cells, the combination of these agents with ganciclovir is much more effective than either agent alone for treating EBV-positive tumors in mouse models (Feng *et al.*, 2002a, 2004). Whether ganciclovir will be effective in combination with lytic induction strategies for treating EBV-positive human tumors is currently being investigated (Faller *et al.*, 2001; Mentzer *et al.*, 2001).

Unresolved issues for the future

The discovery in 1985 that BZLF1 activates lytic EBV expression in latently infected cells was a true milestone in our understanding of lytic EBV gene regulation. Since then, many further advances have been made. However, many questions remain. For example, numerous EBV-encoded microRNAs were recently discovered, including microRNAs which overlap the lytic BHRF1 (anti-apoptosis) and BALF5 (viral DNA polymerase) genes (Shen and Goodman, 2004; Pfeffer *et al.*, 2004) as well as multiple microRNAs located in the introns of the latent BART gene (Cai *et al.*, 2006). Since microRNAs generally promote gene silencing by targeting homologous mRNAs for degradation, the EBV-encoded microRNAs may serve to promote the latent form of viral infection. However, at this point relatively little is

known regarding the regulation of microRNA formation during EBV infection, and whether these microRNAs do indeed regulate viral gene expression. Furthermore, will EBV, like a growing list of other viruses, encode a mechanism for preventing the effect of host genome-derived, antiviral microRNAs?

We still know very little about the nature of the proteins comprising the viral tegument. Does EBV, like other herpesviruses, have a viral tegument protein which serves to transcriptionally activate the viral IE promoters during primary lytic infection? The availability of modern proteomic techniques should allow us to determine the cellular and viral protein composition of the virion, as well as how lytic EBV infection affects cellular proteins. A proteomic analysis of the virion particle was recently published (Johannsen *et al.*, 2004)

It remains unclear which viral proteins confer sensitivity to commonly used antiviral drugs, including acyclovir and ganciclovir. Are these drugs activated primarily by the viral thymidine kinase, or the viral protein kinase (BGLF4)? Alternatively, is the combination of both of these viral proteins required for nucleoside analogue activation in EBV-infected cells? Can we develop new drugs (potentially fatty acid synthase inhibitors) that would inhibit lytic EBV infection at its earliest step, i.e., expression of the viral immediate-early proteins. If we could completely prevent lytic EBV infection in patients (such as organ transplant recipients) who are highly prone to the development of EBV-associated malignancies, would this reduce the number of EBV-induced tumors?

These are only a few of the many questions that will need to be answered by future investigators. What remains certain is that EBV will remain an important and fascinating pathogen for many years to come.

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Reactivation and lytic replication of KSHV

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Overview: goals of lytic replication

Herpesviruses are extremely successful pathogens that have coevolved with their mammalian hosts over the past 60–80 million years (McGeoch and Davison, 1999). This success likely is attributable to the ability of the Herpesviridae to establish lifelong latent infections of their host. Latently infected cells provide a perpetual reservoir from which progeny viruses can be amplified for dissemination within the host and transmission between hosts. Herpesvirologists have traditionally used the term "lytic reactivation" to describe the biological events that begin with emergence of a virus from latency and end with lysis of the host cell and release of progeny virions. Clinico-epidemiologic studies suggest that lytic reactivation of KSHV is an essential pathogenic step in multiple human diseases. The goal of this chapter is to review the host–virus interactions that are critical for regulating induction of KSHV from latency, subsequent progression through the lytic cycle, replication of the viral genome, and assembly of mature viral particles.

Lytic reactivation of KSHV is a critical pathogenic step in development of KS and other human diseases

Numerous epidemiologic studies unanimously agree that reactivation of KSHV from latency is a critical pathogenic step during the progression to KS. Serologic assays (Gao *et al.*, 1996a; Kedes *et al.*, 1996; Martin *et al.*, 1998; Simpson *et al.*, 1996) demonstrate that primary infection by KSHV typically occurs at least 10 years prior to clinically apparent KS in AIDS patients (Martin *et al.*, 1998). During progression of AIDS-KS, seroconversion precedes the first occurrence of detectable viral DNA in peripheral blood (Gao *et al.*, 1996b; Martin *et al.*, 1998; Moore *et al.*, 1996; Whitby *et al.*, 1995), and detection of viral DNA is associated with the appearance of new KS lesions (Cannon *et al.*, 2003). The incidence of KS is tenfold higher in KSHV seropositive patients who have PBMC viremia vs. those without virus in the peripheral blood (Engels *et al.*, 2003; Keller *et al.*, 2001; Moore *et al.*, 1996; Whitby *et al.*, 1995). Furthermore, following KS diagnosis, the amount of KSHV DNA in PBMCs increases with the increased severity of KS staging (Campbell *et al.*, 2000), while lower viral burden in the peripheral blood is associated with a more positive clinical prognosis (Quinlivan *et al.*, 2002). Individual studies of HIV-negative forms of KS (African, Classic or iatrogenic) also concur that KSHV reactivation precedes KS, and viral replication increases during the progression to more severe KS stages (Beyari *et al.*, 2003; Campbell *et al.*, 2003; Cattani *et al.*, 1999; Vitale *et al.*, 2000; Mantina *et al.*, 2001; Boneschi *et al.*, 2001). During KS development, the infection of endothelial cells in skin appears to temporally follow the rise in peripheral blood virus, suggesting that the dermal lymphatics are not a major reservoir for the virus prior to KS (discussed further below).

Lytic replication of KSHV has also been implicated in the pathogenesis of a number of Human diseases other than KS. In AIDS patients with multicentric Castleman's disease (MCD), KSHV viremia in PBMCs fluctuates over a 1 to 2 log range, with peaks corresponding to symptomatic episodes (Oksenhendler *et al.*, 2000; Grandadam *et al.*, 1997). In patients with hemophagocytic lymphohistiocytosis (HL), the peripheral blood load of KSHV is increased at least one order of magnitude during HL events, and frequently precedes these events (Fardet *et al.*, 2003). Methylation of the viral genome that represses the lytic cycle during latency seems to be relieved in patients with KS, MCD, or primary

effusion lymphoma (PEL; described below) (Chen *et al.*, 2001).

The successful treatment of KS patients with drugs that block herpesviral DNA replication suggests that ongoing viral replication is required not only for initiation of KS but for maintenance of the disease. Two such antivirals are: Ganciclovir, a nucleoside analogue that slows the elongation of nascent DNA chains by competitive inhibition of herpesviral DNA polymerase proteins (Field *et al.*, 1983), and Foscarnet, a pyrophosphate analogue that interferes with pyrophosphate exchange between the herpesviral polymerases and deoxynucleoside triphosphates (Wahren *et al.*, 1985). Both drugs reduce KSHV replication in PEL models of infection (Kedes and Ganem, 1997). Independent clinical studies have demonstrated that treatment of high-risk patients with either Ganciclovir (Martin *et al.*, 1999) or Foscarnet (Jones *et al.*, 1995) reduces KS risk, and both drugs slow KS progression if administered subsequent to diagnosis (Robles *et al.*, 1999). Ganciclovir and Foscarnet may cause regression of AIDS-KS without clearance of detectable KSHV from blood (Humphrey *et al.*, 1996), suggesting that viral persistence in the absence of lytic replication is not sufficient to promote KS; however, other treatments have been reported that lead to KS regression accompanied by clearance of peripheral blood viremia (Monini *et al.*, 2001). MCD has also been treated successfully with Ganciclovir, with accompanying reductions in KSHV viremia (Casper *et al.*, 2004a).

The immune system tempers lytic reactivation of KSHV and KS development

The inverse relationship between immunocompetence and the development of KS is most clearly observed in studies of solid organ transplant recipients. More than 50% of KSHV-positive, immunosuppressed organ recipients develop tumors (Farge *et al.*, 1999), and post-transplant KS typically regresses upon cessation of immunosuppression (for review see Hengge *et al.*, 2002). The majority of post-transplant KS patients are KSHV seropositive prior to transplantation, suggesting that reactivation of latent virus is etiologically responsible for the disease (Weigert *et al.*, 2004; Parravicini *et al.*, 1997; Cattani *et al.*, 2001; Andreoni *et al.*, 2001).

Similarly, AIDS-related immunosuppression appears to be a key facet of the contribution of HIV coinfection to KS development and progression. Although KSHV prevalence was 24.6% in 1978 prior to the emergence of HIV infection in San Francisco (Osmond *et al.*, 2002), KS incidence remained low until the AIDS epidemic took hold (Eltom *et al.*, 2002). Detection of KSHV DNA increases, and KS severity worsens, as CD4+ cell counts drop (Keller *et al.*, 2001; Martin, *et al.*, 1998; Min and Katzenstein, 1999; Tedeschi *et al.*, 2001; Whitby *et al.*, 1995). T-cell proliferative responses to KSHV are also reduced, in HIV-1+ and not HIV-1-men and may decrease prior to reduction in CD4+ cell numbers (Strickler *et al.*, 1999). Independent of CD4+ cell count (or HIV-1 infection), antibodies that neutralize KSHV infection are also reduced in KSHV+ people who have KS, as compared to those who are KSHV+ but do not have KS (Kimball *et al.*, 2004). Immune suppression also appears to play a critical role in development of Classic KS (Iscovich *et al.*, 2000; Brenner *et al.*, 2002); however, such patients may live many decades with latent KSHV before KS strikes (if at all) (Cerimele *et al.*, 2000). For example, 13.2% of Sardinian (Italian) children between the ages of 6 and 14 were seropositive for KSHV (Serraino *et al.*, 2000), yet Classic KS is associated with advanced age, with the highest risk for those at least 50 years old (Santarelli *et al.*, 2001).

Immune restoration in AIDS patients treated with highly active antiretroviral therapy (HAART) also implicates the control of KSHV replication by the immune system as a key factor in KS incidence. The introduction of HAART has resulted in recent annual declines of KS of 8.8% to 39% in the USA and Europe (Jones *et al.*, 2000; Mocroft *et al.*, 2004). HAART also decreases progression of AIDS-KS to visceral disease (Nasti *et al.*, 2003), and increases the median time of survival (Tam *et al.*, 2002). During successful HAART, regression of KS and decreasing KSHV detection is accompanied by restoration of numbers and functions of CD4+ cells (Casper *et al.*, 2004b; Lebbe *et al.*, 1998; Mocroft *et al.*, 2004; Wilkinson *et al.*, 2002; Jones *et al.*, 2000; Cattelan *et al.*, 2001; Pellet *et al.*, 2001), CD8+ cells (Bourboulia *et al.*, 2004; Wilkinson *et al.*, 2002), or NK cells (Sirianni *et al.*, 2002). Conversely, HAART non-responders fail to clear KSHV from PBMCs (Sirianni *et al.*, 2002; Cattelan *et al.*, 2001).

However, the success of HAART in ameliorating KS likely is attributable not only to immune restoration, but also to the concomitant reduction in HIV-1 load (Cattelan *et al.*, 2001; Lebbe *et al.*, 1998; Wilkinson *et al.*, 2002). Many studies have documented an increased risk of KS (Cannon *et al.*, 2001; Jacobson *et al.*, 2000) and increased detection of KSHV viremia (Gill *et al.*, 2002; Mantina *et al.*, 2001; Tedeschi *et al.*, 2001) with higher viral loads of HIV-1. Some HAART-treated populations show regression of AIDS-KS that corresponds with decreased HIV-1 loads independently of CD4+ levels (Gill *et al.*, 2002). HIV-1-infected cells produce a soluble factor that induces reactivation of latent KSHV in culture (Varthakavi *et al.*, 1999; Mercader *et al.*, 2000), and

HIV-1 infection of cultured PEL cells also reactivates KSHV (Merat *et al.*, 2002; Varthakavi *et al.*, 1999; Huang *et al.*, 2001). The HIV-1 Tat and Vpr proteins can independently promote lytic cycle induction and replication of KSHV in culture (Huang *et al.*, 2001; Harrington *et al.*, 1997; Merat *et al.*, 2002). HIV protease inhibitors may also block KS independently of the immune system by inhibiting KS cell invasion of the extracellular matrix, in part by blocking matrix metalloproteinase-2 activity (Sgadari *et al.*, 2002).

MHV-68 is a model for immune control of gamma-herpesvirus reactivation from latency

There is currently no small animal model for KSHV infection that recapitulates KS, so it is not possible to scientifically test the host immune response to KSHV infection and the pathogenic consequences in a disease-specific fashion. However, murine gammaherpesvirus (MHV)-68 provides the most compelling experimental evidence of the requirement for a functioning immune system in controlling gammaherpesviral replication, reactivation, and pathogenesis. MHV-68 shares extensive colinearity and conserves many genes with KSHV (Virgin *et al.*, 1997). Intranasal infection of immunocompetent mice leads to primary replicaton in alveolar epithelial cells, followed by establishment of latency in splenic B-cells (Sunil-Chandra *et al.*, 1992a,b). The B-cell reservoir for MHV-68 is CD19+ (Weck *et al.*, 1999a), as is a reservoir of KSHV infection (Dittmer *et al.*, 1999; Ambroziak *et al.*, 1995). A small fraction of latently infected immunocompetent mice proceed to develop lymphoproliferative disease (LPD), but the incidence of LPD can be strongly amplified by treatment of the mice with cyclosporine A (Sunil-Chandra *et al.*, 1994), a calcineurin inhibitor commonly used in humans as an immunosuppressant to prevent rejection of solid organ transplants (for review see Kaufman *et al.*, 2004). This observation has provided the basis for experimental evaluation of MHV-68 pathogenesis in mice with underlying immunodeficiencies generated by various gene-specific knockouts of critical immune effectors.

Mice from many of the respective immunodeficient lines, notably type I interferon null mice (Weck *et al.*, 1997), die during primary, acute MHV-68 infection, before pathogenic analyses of latency and reactivation can be assessed (for review see Simas and Efstathiou, 1998; Speck and Virgin, 1999). However, in mouse lines that do not succumb to the initial viral challenge, both cellular and humoral immunity have been implicated in controlling various characteristics of MHV-68 latency and reactivation. In CD8+ deficient mice, the pool of latently infected cells in the spleen and peritoneum is enlarged, and reactivation efficiency is increased (Tibbetts *et al.*, 2002; Weck *et al.*, 1996; Cardin *et al.*, 1996). Among effector molecules that can be produced by CD8+ T cells, IFNγ and perforin control reactivation efficiency, and the number of latently infected cells, in the peritoneum and in the spleen, respectively (Tibbetts *et al.*, 2002). In mice deficient for major histocompatibility class (MHC) II, MHV-68 establishes a progressive, productive infection, suggesting that CD4+ cells may steer the early infection towards latency (Cardin *et al.*, 1996). Repression of latency can be rescued in this model by treatment of mice with an agonistic antibody to CD40, whose ligand is normally expressed by CD4+ cells in providing help to the antiviral CD8+ cytolytic function (Sarawar *et al.*, 2001). CD4+ cells can also function later during MHV-68 pathogenesis to cause regression of MHV-68 induced B-cell tumors when prestimulated and adoptively transferred to nude mice (Robertson *et al.*, 2001).

Mice that are B-cell deficient as a result of the μ-immunoglobulin knockout (μ-MT) can nonetheless be infected by MHV-68, with latency established most prominently in non-B-cells in the peritoneum (Weck *et al.*, 1999b). In this model, the virus reactivates more efficiently than in healthy mice, and sustains reactivation for greater than 100 days following initial infection, escaping the control of reactivation that normally occurs in healthy mice at 3–4 weeks postinfection (Stewart *et al.*, 1998; Weck *et al.*, 1996, 1999b). The ability of MHV-68 to establish latency in B-cell-depleted mice led to the discovery of a second major reservoir for the virus in macrophages, and therefore to the suggestion that bone marrow may be a source of latently infected macrophages continually available to replenish the periphery (Weck *et al.*, 1999b).

The humoral response to MHV-68 infection also influences latency and reactivation of the virus. CD28-null mice have normal levels of CD4+ and CD8+ T-cells, and B-cells, but their humoral immunity is deficient due to defective T/B-cell collaboration (Shahinian *et al.*, 1993). T-cell depletion by injection of anti-T-cell antibodies in these mice results in reactivation of latent MHV-68, which can be prevented by the prior passive transfer of antiviral immune serum (Kim *et al.*, 2002). In B-cell deficient mice, passive transfer of antilytic cycle antibody, but not antilatent cycle antibody, reduces the frequency of cells harboring latent virus, and the frequency of lytic reactivation of the virus (Gangappa *et al.*, 2002). Combined with the finding that the antiviral drug cidofavir also reduces the frequency of latently infected cells, this suggests that lytic reactivation is required for maintenance of viral persistence (Gangappa *et al.*, 2002).

Experimental infection by MHV-68 has also proven powerful for differentiation of true latent infection from chronic/persistent infection, a distinction that is very difficult to ascertain in natural Human infections by gammaherpesviruses. MHV-68-specific immunoglobulin (Ig) G remains elevated after peaking 2 weeks following infection (Stevenson and Doherty, 1998), perhaps as a result of intermittent reactivation of latent virus (Simas *et al.*, 1999). A similar elevation of virus-specific antibodies has been described for KSHV that is sustained for many years following seroconversion in HIV+ and HIV− men (Gao *et al.*, 1996a; Biggar *et al.*, 2003), suggesting that intermittent reactivation from latency, or persistent replication of KSHV, may occur in certain hosts. Chronic replication of MHV-68 primarily leads to smooth muscle infection of large vessels and arteritis, which occurs with different frequencies in mice lacking an interferon gamma response (type II interferon), functional B-cells, functional CD4+ cells, or MHC II (Dal Canto *et al.*, 2002; Weck *et al.*, 1997).

Sites of latency and reservoirs for viral amplification in vivo

All of the gammaherpesviruses can infect lymphoid cells, and all can produce disease in that lineage. KSHV can also establish latency in non-lymphoid cells, but each site appears to make unique contributions to KSHV pathogenesis, and not all sites appear to be significant long-term reservoirs of amplifiable virus.

As described earlier in this chapter, after a KSHV-infected individual seroconverts, the titer of viral DNA in peripheral blood varies in direct proportion to the speed of progress to KS and severity of subsequent disease. The peripheral blood reservoir for KSHV has been unanimously identified as CD19+ B-cells, both prior to, or during, KS and in MCD patients. (Ambroziak *et al.*, 1995; Bigoni *et al.*, 1996; Blackbourn *et al.*, 1997; Harrington *et al.*, 1996; Kikuta *et al.*, 1997; Whitby *et al.*, 1995). Infected B-cells have also been localized to lymph nodes (Dupin *et al.*, 1999; Schalling *et al.*, 1995), and can support both latent and lytic gene expression (Polstra *et al.*, 2003). Virus has also occasionally been detected in peripheral T-cells and other monocytes (Blasig *et al.*, 1997; Harrington *et al.*, 1996; Kikuta *et al.*, 1997; Sirianni *et al.*, 1997), and monocytes can maintain the viral infection if cultured in vitro in the presence of inflammatory cytokines (Monini *et al.*, 1999b). CD19 negative, cultured PEL specimens also support latent and lytic infection; moreover, PEL cells release mature virus that can infect primary CD19+ cells in culture (Renne *et al.*, 1998), and that can establish long-term latency in CD19+ B-cells in human thymus/liver (thy/liv) tissue implanted into mice with severe-combined immunodeficiency (SCID) (Dittmer *et al.*, 1999). Thus B-cells are regarded as one site of long-term KSHV latency that can serve as a reservoir of virus for subsequent dissemination throughout the body.

Endothelial cells are a second site of latency for KSHV, and are associated with a much higher frequency of pathology than other cells infected by the virus. In KS tumors, the KSHV-infected cell is a CD34+ endothelial cell with characteristic spindloid morphology (Boshoff *et al.*, 1995; Staskus *et al.*, 1997). However, endothelial cells do not appear to be a long-term latent reservoir for the virus that can provide a significant source of progeny virions. KSHV DNA is infrequently detected in non-diseased skin of KS patients (Nuovo and Nuovo, 2001; Boshoff *et al.*, 1995; Chang *et al.*, 1994; Moore and Chang, 1995) and the virus is usually not detected in skin following regression of lesions (Aluigi *et al.*, 1996; Ambroziak *et al.*, 1995). Furthermore, the number of viral copies per cell appears to be at least one order of magnitude lower in endothelial cells than in other latently infected cells (Boshoff *et al.*, 1995; Chang *et al.*, 1994). Although the dermal endothelium is probably not a long-term latent reservoir, infected spindle cells may release progeny virus that can subsequently infect local keratinocytes and eccrine epithelium in the tumor (Reed *et al.*, 1998).

A significant question for KSHV reactivation and pathogenesis concerns the mechanism by which the virus disseminates from the peripheral blood reservoir to sites of KS development. A strong argument for direct seeding of putative tumor sites by infected B-cells comes from tissue culture studies that show: (i) promotion of specific arrest and *trans*endothelial migration of KSHV-infected B-cells in response to the chemoattractant stromal derived factor (SDF)-1 (Yao *et al.*, 2003), and (ii) transmission of virus from stimulated B (PEL) cells to primary endothelial cells (Sakurada *et al.*, 2001). However, KSHV-infected mononuclear cells have only been detected in some (Blasig *et al.*, 1997; Aluigi *et al.*, 1996; Reed *et al.*, 1998; Sirianni *et al.*, 1998) but not all (Pyakurel *et al.*, 2004; Staskus *et al.*, 1997), histopathologic samples of KS tissue. Furthermore, among the studies that have detected KSHV-infected mononuclear cells in tumors, the immunophenotype of the cells has been described variably as either lymphocytic (Reed *et al.*, 1998), or monocytic/macrophagic (Blasig *et al.*, 1997; Pyakurel *et al.*, 2004; Sirianni *et al.*, 1998). Therefore, although early KS lesions contain readily detectible lymphocytic and plasma cell infiltrates (Niedt *et al.*, 1990), the peripheral blood reservoir of the virus may not directly, or continually, seed the tumor. An alternative mechanism may be that KSHV-infected, CD34+ spindle cells circulating in

peripheral blood (Henry *et al.*, 1999; Browning *et al.*, 1994; Sirianni *et al.*, 1997, 1998) may be endothelial precursor cells (Asahara *et al.*, 1997) that subsequently populate the tumors.

While the peripheral blood probably maintains the long-term cellular reservoir of KSHV that can serve as a source of progeny virus for dissemination within an individual, a second, oral/salivary reservoir appears to be critical for producing high titer virus that can be transmitted between individuals. In fact, the titer in saliva is higher than in PBMCs (LaDuca *et al.*, 1998; Koelle *et al.*, 1997; Pauk *et al.*, 2000), and viral DNA can be detected in saliva in individuals with undetectable PBMC virus (Blackbourn *et al.*, 1998). The virus appears to maintain latency in oral epithelium (Triantos *et al.*, 2004; Pauk *et al.*, 2000), but significant amounts of encapsidated KSHV can be found free of cells in salivary fluid (Vieira *et al.*, 1997; Blackbourn *et al.*, 1998), and are transmissible to cultured cells (Duus *et al.*, 2004; Vieira *et al.*, 1997). Thus, reactivation of virus from latently infected cells in the oral cavity may provide the natural source of KSHV for inter-personal transmission. However, direct transmission of cells infected by KSHV cannot be excluded, as this mechanism has been reported in some cases of post-transplant KS (Barozzi *et al.*, 2003; Luppi *et al.*, 2003).

Primary effusion lymphoma (PEL) cells: a tissue culture model for KSHV latency and reactivation

Within the family *Herpesviridae*, the most robust systems for understanding molecular events in latency and reactivation are tissue culture models of infection by the gamma-2-herpesviruses like KSHV (Cesarman *et al.*, 1995, 1996; Nador *et al.*, 1996; Renne *et al.*, 1996a,b). The first model systems for understanding KSHV latency and reactivation have been cells cultured from clinical specimens of primary effusion lymphoma (PEL). PEL is the only B-cell lymphoma conclusively associated with KSHV infection (Drexler *et al.*, 1998; Cesarman *et al.*, 1995; Uphoff *et al.*, 1998), and is pathologically and phenotypically unique among all non-Hodgkin's lymphomas (Nador *et al.*, 1996). Comparison of all HIV-associated lymphomas shows that PELs are BCL-6-/MUM1+/syn-1+, an immunophenotype that is unique to PEL among all serous effusions (Carbone *et al.*, 2000, 2001) but is shared with EBV-transformed cells that express LMP-1 (Carbone *et al.*, 2001).

Cytogenetic and gene expression analyses classify PEL cells as preterminally differentiated, postgerminal center B-cells, with overall similarities to plasma cells. Although PELs are immunophenotypically non-B/non-T-cells, their B-cell origin is revealed by clonal rearrangements of heavy and light chain immunoglobulin (Ig) genes that contain mutations suggestive of antigen selection (Fais *et al.*, 1999). Genome-wide expression profiling using microarrays has confirmed that PELs cluster phenotypically with transformed plasma cells, EBV lymphoblastoid cell lines, and multiple myeloma cells, but nonetheless remain a distinct NHL subset (Jenner *et al.*, 2003; Klein *et al.*, 2003). PEL cell lines are transformed (Picchio *et al.*, 1997; Strauchen *et al.*, 1996; Gaidano *et al.*, 1996; Boshoff *et al.*, 1998), and most PELs are coinfected with KSHV and EBV (Drexler *et al.*, 1998); however, the existence of EBV-negative PEL lines suggests that KSHV infection is sufficient for transformation of B cells. In fact, the cellular gene expression profiles of PELs singly infected with KSHV, or doubly infected with KSHV and EBV, are indistinguishable (Jenner *et al.*, 2003; Klein *et al.*, 2003).

During normal maintenance and passage, the PEL cells are infected uniformly with 40–150 episomal copies of clonal virus per cell genome (a characteristic of each line (for review see Drexler *et al.*, 1998), with the virus remaining circularized and nuclear (Renne *et al.*, 1996a). Latency is distinguished by highly restricted viral gene expression and lack of mature virus production (although a characteristic 1–5% of each cell line's population undergoes spontaneous reactivation). Upon treatment with various stimuli, including phorbol ester (i.e., TPA) or histone deacetylase inhibitors (i.e., sodium butyrate or trichostatin A), viral lytic genes are induced in a cascade fashion, leading to viral replication and progeny virus production (Renne *et al.*, 1996a,b; Zhong *et al.*, 1996; Miller *et al.*, 1997). As in the other gamma-herpesviruses, viral DNA is packaged into enveloped virions as a 160–170 kilo-basepair (kbp) linear molecule (Renne *et al.*, 1996a).

Kinetic classification of KHSV lytic gene expression

In tissue culture and clinical samples, KSHV exhibits both latent (non-productive) and lytic (productive) replication, which are characterized by virtually distinct gene expression programs (Renne *et al.*, 1996b; Zhong *et al.*, 1996; Miller *et al.*, 1997; Staskus *et al.*, 1997). The formal classification of the kinetics of expression of the lytic genes following treatment of latent virus with inducing agents has been defined for immediate–early (IE) and late genes.

Transcription of IE genes requires no previous protein expression, as evidenced by resistance to protein synthesis inhibitors like cycloheximide (CHX). In herpesviral reactivation systems, the IE genes are the prime candidates to

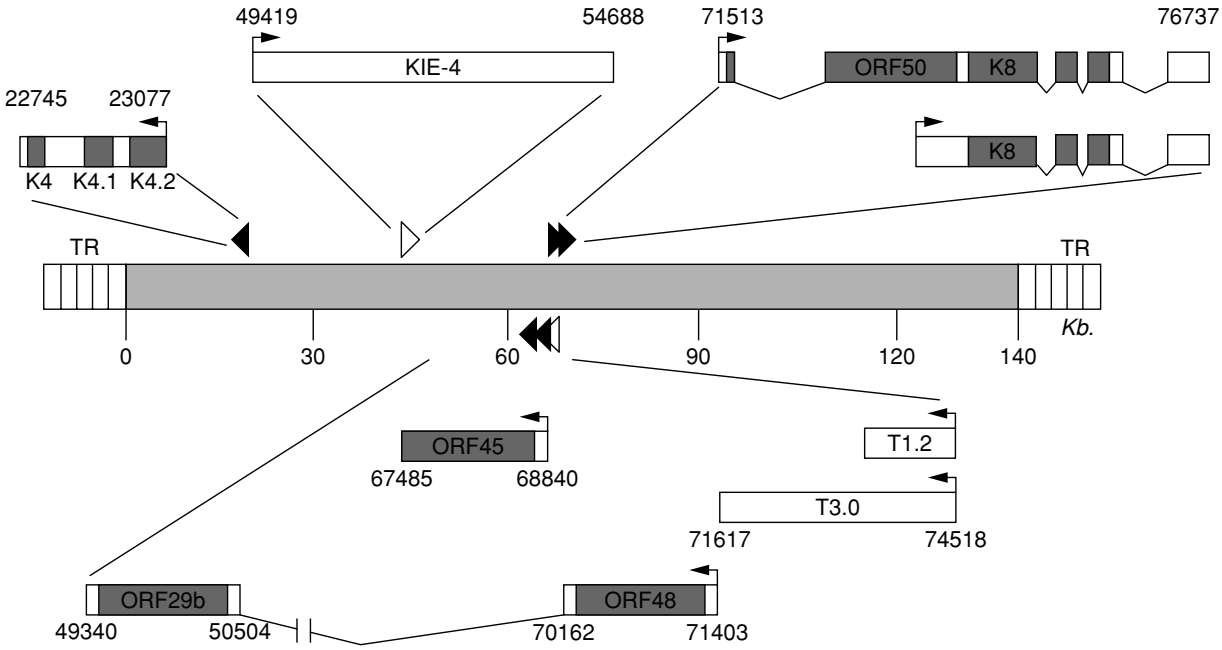

Fig. 26.1. The immediate–early (IE) loci of KSHV. This schematic represents the genome of KSHV. Arrowheads depict the relative locations and directions of IE gene expression. Each is expanded to show the details of transcript architecture. Each box represents a transcript, shading indicates open reading frames, non-shaded indicates untranslated regions, lines indicate introns. Names of each transcript and corresponding ORF are indicated. K8 is expressed in alternatively spliced, mono- and bi-cistronic transcripts; only one of K8's alternatively spliced products is shown here, for simplicity. "TR" = terminal repeat of virus. Numbering refers to nucleotide position on genome (as in Russo *et al.*, 1996). References can be found in the text.

function as master controllers of the latent-to-lytic switch, a characteristic conserved in KSHV (discussed below). Eight distinct transcripts that are expressed in an IE fashion during KSHV reactivation encode ORF50/K8α/K8.2, K8α, ORF45, K4.2/K4.1/K4, ORF48 and ORF29b, K3 and ORF70, and two with no apparent coding potential (Fig. 26.1) (Wang *et al.*, 2004b; Zhu *et al.*, 1999a; Saveliev *et al.*, 2002; Rimessi *et al.*, 2001). Transcription of the L genes requires prior viral DNA replication, and the L transcriptome has been defined using the replication inhibitor cidofovir (CDV), a nucleoside analog (Lu *et al.*, 2004). The study showed that expression of all of the known virion structural genes was inhibited by prior treatment of PEL cells with CDV. However, only about 25% of the viral membrane glycoproteins were inhibited by CDV. Most of the lytic cycle homologs of cellular genes were insensitive to CDV, except for the viral interferon regulatory factors vIRF-2 and vIRF-3/LANA2, viral macrophage inflammatory protein (vMIP)-III, and the viral FLICE inhibitory protein (vFLIP). Also insensitive to CDV were the IE transactivators and all of the known viral replication proteins. By default, lytic genes that have not been classified as IE or L fall into the delayed early (DE) class.

Genome-wide approaches have been used to classify KSHV transcription based on (i) quantity of expression in the presence or absence of TPA (Sarid *et al.*, 1998), or (ii) time of first appearance and peak level of expression (Fakhari and Dittmer, 2002; Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001). These studies have generally agreed that (i) the latent genes are typically expressed regardless of TPA addition, (ii) putative regulatory genes are expressed weakly prior to TPA addition, but strongly within 10 hours postinduction (pi), (iii) genes for putative DNA repair and nucleotide metabolism proteins are only expressed 10–24 hours pi, and (iv) genes for virion formation and structural proteins are expressed 48–72 hours pi. The mechanisms regulating coordinate induction of expression of most of the lytic genes during reactivation have not been evaluated in a systematic fashion.

ORF50/Rta is the viral lytic switch protein

Among a number of candidate KSHV genes, only transfection of Rta (the product of the ORF50 gene) can induce lytic reactivation of the virus in PEL cells, as detected by

Northern and Western blotting, and immunofluorescence (Lukac *et al.*, 1998; Sun *et al.*, 1998). Induction of the ORF59 (DE) and K8.1 (L) proteins by Rta transfection is quantitatively equivalent to TPA treatment, and K8.1 induction is blocked by PFA treatment, suggesting that Rta induces authentic viral DNA replication (Lukac *et al.*, 1998; Polson *et al.*, 2001). Productive release of encapsidated KSHV virions in response to forced expression of Rta confirms that Rta induces the entire lytic gene cascade (Gradoville *et al.*, 2000; Nakamura *et al.*, 2003).

ORF50 encodes a 691 amino acid protein that is a direct and selective transcriptional transactivator of DE promoters in uninfected cells (Lukac *et al.*, 1998). Rta shares with its homologues in the gamma-herpesviruses two regions of relatively high primary amino acid homology, in the far N- and C-termini (Lukac *et al.*, 1998). The N-terminal 272 amino acids can bind independently to KSHV promoter DNA (Lukac *et al.*, 2001; Song *et al.*, 2002), and the C-terminus is a potent transactivator when targeted to promoters by fusion to heterologous DNA binding domains (Lukac *et al.*, 1999; Seaman *et al.*, 1999; Wang *et al.*, 2001). Deletion of the activation domain generates a mutant of Rta that forms mixed multimers with wild-type Rta and functions as an ORF50-specific dominant negative inhibitor of transactivation (Lukac *et al.*, 1999). Expression of this dominant negative Rta mutant in PEL cells suppresses viral reactivation induced by TPA, sodium butyrate, and ionomycin (Lukac *et al.*, 1999). Therefore, transcriptional transactivation by Rta is both sufficient and necessary for viral reactivation, and multiple reactivation signals that function using different biochemical mechanisms all converge at ORF50/Rta to successfully reactivate the virus (Lukac *et al.*, 1999).

ORF50/Rta binds directly to the DNA of several KSHV promoters (Fig. 26.2) with varying affinity and sequence-specificity. The relative binding strengths for Rta to these promoter elements have been estimated as PAN>kaposin>ORF57>vIL-6 (Song *et al.*, 2003), with a dissociation constant (K_d) for binding to the PAN element in the nanomolar range (Song *et al.*, 2002). The PAN and kaposin promoters share a 16-bp core sequence that is also found in *ori-Lyt* (L) (Wang *et al.*, 2004b; Kirshner *et al.*, 2000; Song *et al.*, 2002; Chang *et al.*, 2002), but differs significantly from a second DNA element shared by the ORF57/Mta and K8 promoters (Lukac *et al.*, 2001), and a third element in the viral IL-6 promoter (Deng *et al.*, 2002).

However, direct DNA binding by Rta to all of these promoters is insufficient to specify them as targets for transactivation; instead, Rta requires combinatorial interactions with cellular proteins. Scanning mutagenesis of the Rta-responsive elements of both the PAN and Mta promoters demonstrated incomplete concordance between DNA binding by Rta and transactivation, i.e., some mutants that failed to bind Rta remained strongly transactivated by Rta, and vice versa (Song *et al.*, 2002; Lukac *et al.*, 2001). Furthermore, fusion of Rta's minimal DNA binding domain (Song *et al.*, 2002; Lukac *et al.*, 2001) to a heterologous activation domain is insufficient to transactivate the PAN promoter in vivo, but requires additional Rta amino acids that likely interact with heterologous proteins (Chang *et al.*, 2002).

To this end, Rta transactivation requires cooperative interactions with various cellular transcription factors (Fig. 26.2). Genetic and biochemical experiments demonstrate that *trans*-activation of the ORF57/Mta promoter by Rta requires a direct interaction with the sequence-specific DNA binding protein RBP-Jk (aka CBF-1 and CSL). RBP-Jk-binding sites also mediate Rta-driven activation of the promoters of KSHV ORF6/single-stranded DNA binding (SSB) protein, the viral GPCR, and of Rta itself (auto-activation) (Liang and Ganem, 2003, 2004; Liang *et al.*, 2002). A central regulatory role of the RBP-Jk/Rta interaction was demonstrated by the inhibition of KSHV reactivation in murine embryo fibroblasts null for RBP-Jk (Liang and Ganem, 2003). Rta promotes DNA binding of RBP-Jk, a mechanism that is fundamentally different from that established for the RBP-Jk-activating proteins, Notch intracellular domain (NICD) and EBV EBNA-2 (Carroll *et al.*, 2006). Cooperation between RBP-Jk and Rta requires intact DNA binding sites for both proteins, and trimeric complex formation between the three molecules in vitro. In infected cells, chromatin immunoprecipitations reveal that RBP-Jk is virtually undetectable on the viral ORF57, K-bZIP, vGPCR, and cellular IL-6 and hairy/enhancer of split (HES)-1 promoters (Carroll *et al.*, 2006). These data provide one explanation for the ability of KSHV to establish latency in RBP-Jk null cells (Liang and Ganem, 2003). However, during viral reactivation. RBP-Jk is significantly enriched on all of those promoters in an Rta-dependent fashion. Accordingly, Rta, but not EBNA2 and NICD, reactivates the complete viral lytic cycle. KSHV might have evolved this mechanism to ensure that only Rta efficiently reactivates the virus in environments of consititutive Notch activity.

Rta also participates in complex, combinatorial interactions with the cellular protein C/EBPα for transient transactivation of various KSHV promoters (Fig. 26.2). Rta and C/EBPα cooperate to activate transcription of the K8, ORF57/Mta, ORF50/Rta, and nut-1/PAN promoters, each of which contains at least one binding site for C/EBPα (Wang *et al.*, 2003a,b). A direct interaction between C/EBPα and Rta is required for transcriptional cooperation (Wang

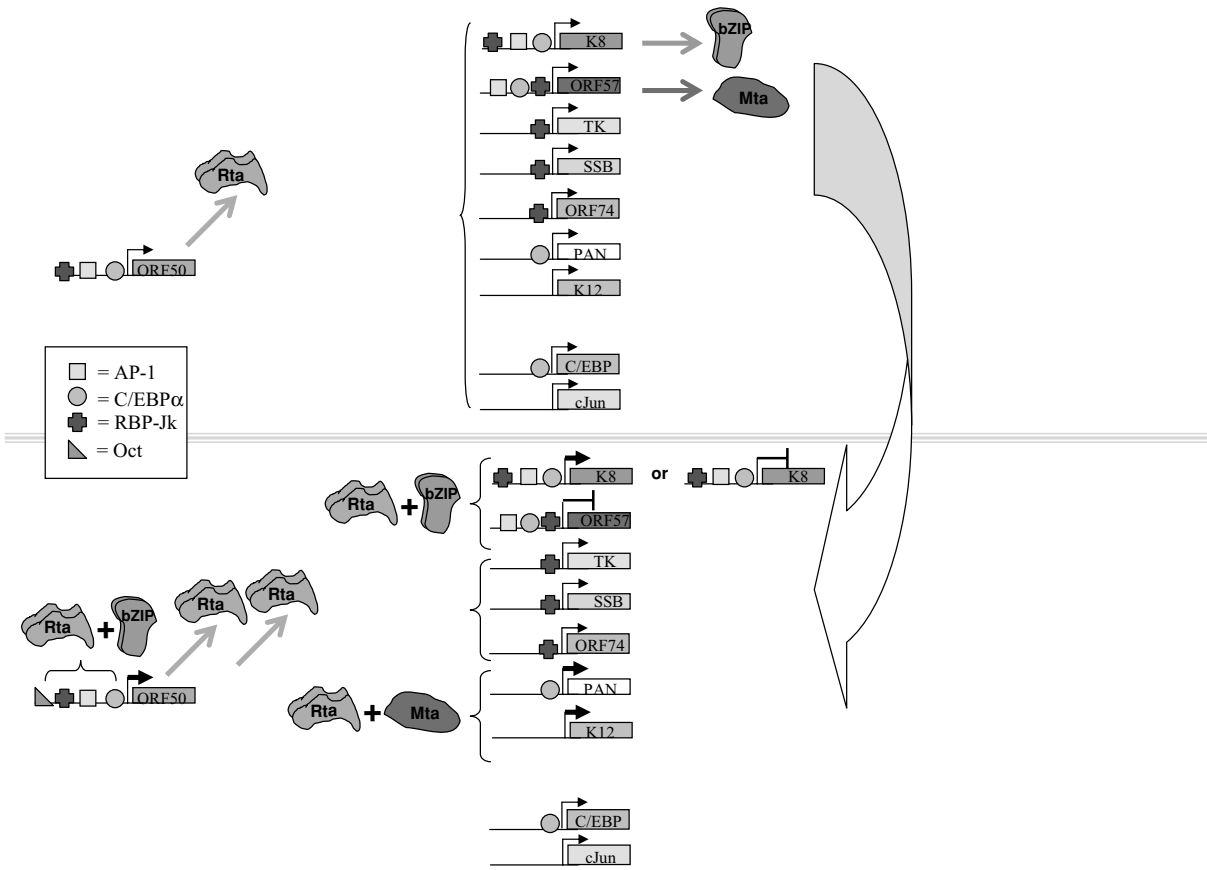

Fig. 26.2. Rta is the KSHV lytic switch protein. The viral and cellular promoters transactivated by Rta. *Cis* elements are indicated by symbols placed on each promoter, and *trans*-acting factors are indicated by the colored arrows. The size of the arrow at the transcriptional start site reflects relative amounts of transcription. See text for details.

et al., 2003a,b), and the C/EBPα binding sites overlap the Rta binding sites in the Mta and K8 promoters (Lukac *et al.*, 2001). In infected PEL cells, C/EBPα and Rta are found associated with the Rta, PAN, or Mta promoters only following viral reactivation (Wang *et al.*, 2003b); immunodepletion of C/EBPα prior to chromatin immunoprecipitation (ChIP) eliminates Rta's association with the Rta promoter, and reduces its association with the K8 promoter. Data also suggest that Rta cooperates with C/EBPα in auto-activation of the C/EBPα promoter, such that Rta and C/EBPα mutually participate in a positive autoregulatory loop during reactivation of KSHV from latency (Wang *et al.*, 2003a,b).

Rta and the cellular protein interferon regulatory factor (IRF)-7 participate in another regulatory loop. An IRF-7 binding site overlaps the Rta-responsive element in the ORF57 promoter, and IRF-7 inhibits Rta transactivation by competitive DNA binding to the element (Wang *et al.*, 2005). Rta contains intrinsic E3 ubiquitin ligase activity that targets IRF-7 for degradation by the host proteasome (Yu *et al.*, 2005). Interestingly, Rta also autoregulates its own degradation by self-ubiquitination (Yu *et al.*, 2005).

Rta also cooperates with other cellular proteins in promoter association and transactivation. cJun and cFos interact directly with each other and Rta, and all three cooperate to transiently transactivate the K8, Mta, and Rta promoters in uninfected cells (Wang *et al.*, 2004). Exogenous expression of Rta induces expression of cJun in BCBL-1 cells, and cJun coprecipitates with the K8, Mta, and Rta promoters in TPA-treated, but not untreated, BCBL-1 cells (Wang *et al.*, 2004). Rta and cJun may also cooperate through a mutual interaction with the coactivator CBP (Gwack *et al.*, 2001a). Rta can also be directed to autoactivate its own promoter through interactions with the cellular protein Octamer-1, but not Octamer-2, and an Octamer DNA element near the ORF50 transcription start site (Sakakibara *et al.*, 2001). Activation of the K9/vIRF promoter by Rta appears to depend exclusively upon unknown cellular DNA binding factors and not by direct Rta binding (Ueda *et al.*, 2002).

Nonetheless, DNA binding by Rta is obligatory for transactivation of the Rta-responsive elements of the PAN and Kaposin promoters (Chang *et al.*, 2005b).

The promoter association of Rta is also regulated by post-translational modifications and homotypic interactions. Poly-ADP-ribosylation and phosphorylation of Rta's serine–threonine (ST)-rich region (AAs 505–520) decreases Rta's interactions with the Rta and Mta promoters in reactivating BCBL-1 cells (Gwack *et al.*, 2003b). The cellular proteins poly(ADP-ribose) polymerase-(PARP)-1, and the human homologue of kinase from chicken (KFC), interact directly with the ST-rich region of Rta to add these modifications, elimination of which enhances Rta-mediated transactivation and viral reactivation (Gwack *et al.*, 2003b). Other data show that Rta oligomerizes to bind the K8 promoter in vitro (Liao *et al.*, 2003). All of the above data suggest that Rta expression alone may not be sufficient to reactivate KSHV, but additional levels of control following Rta expression are also essential. Indeed, in single cell reactivation assays, less than 10% of Rta-transfected PEL cells express viral lytic proteins (Lukac *et al.*, 1998, 1999). Progression down the lytic gene cascade may therefore be inefficient, an observation reported, as well, in infection of cultured endothelial cells (Ciufo *et al.*, 2001).

After Rta associates with lytic KSHV promoters, its ability to reactivate the virus from latency depends upon successful recruitment of the cellular SWI/SNF chromatin remodeling complex, and the TRAP/mediator coactivator (Gwack *et al.*, 2003a). Direct contacts of Rta to individual proteins in both complexes is required for Rta-mediated transactivation, and Rta presumably recruits the remaining members in stoichiometric complexes to mediate these effects (Gwack *et al.*, 2003a). In the case of promoters that require interactions of Rta with RBP-Jk (Liang *et al.*, 2002; Liang and Ganem, 2004; Lukac *et al.*, 2001), this suggests that Rta can replace the role of the activated cellular Notch protein in recruiting histone acetyl-transferase (HAT) complexes to RBP-Jk dependent promoters.

Rta's activity is also modulated by complex interactions with a second KSHV IE protein, K8/KbZIP (also known as RAP). Unlike its homologue in EBV, the Zta protein, KSHV KbZIP is unable to reactivate the KSHV lytic cycle (Polson *et al.*, 2001). Instead, stable expression of the K8/KbZIP protein in BCBL-1 cells inhibits replication of KSHV in response to TPA induction (Izumiya *et al.*, 2003a). This function may be due to the ability of K8/KbZIP to inhibit Rta-mediated transactivation of the ORF57/Mta and K8 promoters (Liao *et al.*, 2003; Izumiya *et al.*, 2003a). The K8/KbZIP protein directly targets the transcriptional coactivator CREB-binding protein (CBP) to transcriptionally repress signaling induced by either TPA or TGFβ, as well as the cFos promoter (Hwang *et al.*, 2001; Tomita *et al.*, 2004). However, the ability of K8/KbZIP to repress Rta-mediated activation requires a direct interaction between itself and Rta, and is not relieved by overexpressing transcriptional coactivators (Liao *et al.*, 2003).

There are also conditions in which K8/KbZIP activates transcription. K8/KbZIP can cooperate with Rta to transactivate the K8 and Rta promoters (Wang *et al.*, 2003a), but does not affect Rta-mediated activation of the nut-1/PAN promoter (Izumiya *et al.*, 2003a). Collectively, these data suggest that K8/KbZIP may cooperate with Rta to ensure strong induction of Rta expression, but then tempers lytic cycle progression by inhibiting Rta's ability to activate downstream promoters, like that of ORF57 and K8. As described below, K8/ KbZIP also inhibits cell cycling, so the mechanisms by which K8/ KbZIP influences Rta function are likely to be complex.

Other viral proteins exert both positive and negative effects on Rta. A DE target of ORF50/Rta transactivation, ORF57/Mta, is essential for infectious virion production from latently infected cells (Han and Swaminathan, 2006). The Mta ORF encodes protein domains with putative transcriptional and post-transcriptional functions, and stimulates the accumulation of viral lytic mRNAs (Han and Swaminathan, 2006). Mta uses an un-characterized mechanism to synergize with Rta in a promoter-specific fashion (Kirshner *et al.*, 2000). The ORF73 protein, Latency-Associated Nuclear Antigen (LANA)-1, represses both Rta-mediated transactivation and reactivation, as well as Rta expression, through interactions with the cellular protein RBP-Jk (Lan *et al.*, 2005).

Signals that control lytic reactivation of KSHV

Various laboratory chemicals are the strongest inducers of the KSHV lytic cascade in PEL cells, and have provided important clues regarding the intracellular pathways that control viral reactivation. Since these chemicals function by divergent mechanisms, it suggests that more than one pathway is sufficient to reactivate KSHV. However, the candidate physiologic ligands that stimulate these pathways are virtually unknown (Fig. 26.3).

TPA has been the standard reagent that induces the entire lytic gene cascade and release of mature progeny virus from PEL cells (Renne *et al.*, 1996b; Miller *et al.*, 1997). TPA added to PEL cells rapidly induces the DNA binding of the cellular proteins C/EBPα and cJun to the Rta promoter; in the absence of TPA, transfection of expression vectors for C/EBPα or the AP-1 constituents cFos and cJun induce Rta expression (Wang *et al.*, 2003a,b, 2004). Newly

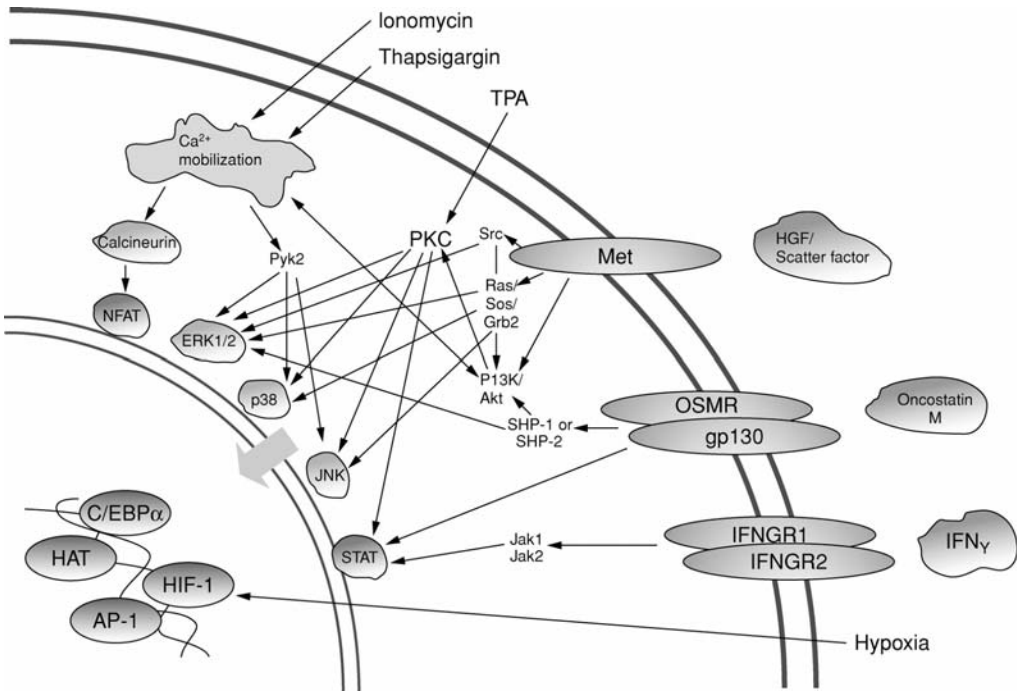

Fig. 26.3. Signals that reactivate KSHV in PEL models of latency. Published or hypothetical pathways that induce KSHV reactivation in PEL cells; see text for details and citations (for review see Maulik *et al.*, 2002; Taga and Kishimoto, 1997; Ernst and Jenkins, 2004; Leonard and O'Shea, 1998; Feske *et al.*, 2003; Yang and Kazanietz, 2003). Abbreviations: "NFAT" = nuclear factor of activated T cells, "TPA" = 12-O-tetradecanoyl-phorbol-13- acetate, "PKC" = protein kinase C, "HGF" = hepatocyte growth factor, "PI3K" = phospho-inositol-3-kinase, "JNK" = jun N-terminal kinase, "OSMR" = oncostatin M receptor, "SHP" = src homology domain-2 domain-containing protein tyrosine phosphatase, "IFN" = interferon, "IFNGR" = interferon gamma receptor, "Jak" = janus kinase, "STAT" = signal transducer and activator of transcription, "C/EBP" = CCAAT/enhancer binding protein, "HAT" = histone acetyl-transferase, "HIF" = hypoxia induced factor.

expressed Rta subsequently induces additional C/EBPα and cJun expression, and both proteins are detected in increasing amounts on DE promoters (Wang *et al.*, 2003a,b, 2004). Chemical inhibitors of protein kinase C (PKC) reduce TPA-induced lytic reaction of KSHV in PELs by 70–75% (Zoeteweij *et al.*, 2001). Collectively, these data suggest that TPA reactivates KSHV primarily by activating AP-1 and C/EBPα in the nucleus through induction of protein kinase C activity. As shown in Fig 26.3, PKC sits at the crossroads of many candidate physiologic ligands and downstream effectors (for review see Yang and Kazanietz, 2003), suggesting that it probably plays a prominent role in controlling KSHV reactivation in vivo.

Although stimulation of PKC can also lead to activation of the NF-kB family of transcription factors (Yang and Kazanietz, 2003), NF-kB is constitutively active in PELs in the absence of TPA. Rta and other lytic proteins are induced if NF-kB is inhibited, resulting in reactivation of KSHV (Brown *et al.*, 2003). Transfection of an expression vector for p65, a transcriptionally active subunit of NF-kB, inhibits the ability of Rta to transactivate its own promoter, and that of nut-1/PAN. This effect is overcome by overexpression of a dominant negative inhibitor of NF-kB (Brown *et al.*, 2003). The latently expressed KSHV protein, vFLIP (viral fas-ligand IL-1 β-converting enzyme inhitibory protein), maintains constitutive activity of NF-kB in PELs, inhibition of which induces apoptosis (Ghosh *et al.*, 2003; Keller *et al.*, 2000). Evolutionary pressure might thus have selected this function of vFLIP to enhance the stable maintenance of KSHV latency.

Calcium ionophores also reactivate KSHV from latency in PEL cells (Renne *et al.*, 1996b; Chang *et al.*, 2000), but are blocked by chemical inhibitors of calcineurin, suggesting a PKC-independent pathway downstream of Ca^{2+} (Yang and Kazanietz, 2003; Zoeteweij *et al.*, 2001). Typically, calcineurin stimulation activates the nuclear factor of activated T cells (NFAT) growth control pathway (for review see Feske *et al.*, 2003). However, NFAT signaling alone is

apparently insufficient to reactivate KSHV: the viral K1 protein, a constitutive activator of NFAT (Lagunoff *et al.*, 1999; Lee *et al.*, 1998), is incapable of inducing reactivation when ectopically overexpressed in PEL cells (Lukac *et al.*, 1998). A mutant of K1 unable to stimulate NFAT blocks reactivation of KSHV induced by Rta, suggesting that NFAT stimulation is essential for events downstream of Rta (Lagunoff *et al.*, 2001). Overexpression of wild-type K1 can also inhibit reactivation without altering Rta expression (Lee *et al.*, 2002), suggesting that NFAT activation must be "fine-tuned" during lytic cycle progression.

Many of the inflammatory cytokines and other soluble factors found in KS lesions (for review see Ensoli *et al.*, 2001) can reactivate the virus if added to the growth media of PEL cells. These include oncostatin M, hepatocyte growth factor, interferon γ, HIV-1 Tat, and an unidentified soluble factor released from HIV-1 infected cells (Blackbourn *et al.*, 2000; Chang *et al.*, 2000; Huang *et al.*, 2001; Mercader *et al.*, 2000; Monini *et al.*, 1999b; Varthakavi *et al.*, 1999). Furthermore, many of these factors are required for growth of uninfected, explanted KS spindle cells (Cai *et al.*, 1994; Ensoli *et al.*, 1989; Miles *et al.*, 1992; Naidu *et al.*, 1994), suggesting that reactivation of KSHV may be co-regulated with stimulation of putative target cells. Supporting this hypothesis, the persistence of KSHV in explanted PBLs from infected humans requires the addition of inflammatory cytokines to the culture (Ensoli *et al.*, 2000; Monini *et al.*, 1999b; Sirianni *et al.*, 1998). Similarly, MHV-68 infection induces the pro-inflammatory cyclooxygenase (COX)-2 protein, and COX-2 inhibitors globally suppress MHV-68 gene expression and virion production (Symensma *et al.*, 2003). However, not all inflammatory cytokines augment KSHV replication: interferon α inhibits reactivation of KSHV in PELs, and reduces the viral load in cultured PBLs (Monini *et al.*, 1999a).

Growth of PELs in hypoxia (limited O_2) results in the accumulation of the cellular transcription factor HIF-1 and also reactivates KSHV (Davis *et al.*, 2001). The messages for Rta and ORF34 are induced in hypoxia, and both contain putative hypoxia response elements (HREs) in their promoters (Haque *et al.*, 2003). Using reporter plasmids for the two viral promoters, the effect of hypoxia is also seen in uninfected cells, and can be reproduced by transfection of expression vectors for HIF-1 and HIF-2 (Haque *et al.*, 2003). These data are provocative considering that (i) classic KS has a predilection to affect the lower extremities of elderly men (Hengge *et al.*, 2002), which often have relatively low tissue oxygen concentrations (Ubbink *et al.*, 1997), and (ii) endemic KS is highly prevalent in areas of the world with concurrent malaria or common hereditary anemias (Sarid *et al.*, 1999), which also lead to tissue hypoxia. Thus, hypoxia may favor reactivation of latent virus, increased viral titer, and consequently, increased KS or increased transmission of KSHV.

Many studies suggest that KSHV reactivation signals ultimately lead to relief of transcriptionally repressive chromatin architecture by increasing acetylation and chromatin remodeling of viral promoters, especially that of Rta. Histone deacetylase (HDAC) inhibitors, like sodium butyrate, trichostatin A, and valproic acid (Lu *et al.*, 2003; Miller *et al.*, 1997; Renne *et al.*, 1996b; Shaw *et al.*, 2000; Vieira and O'Hearn, 2004), all reactivate KSHV from latency, and can directly activate Rta's promoter (Lu *et al.*, 2003). ChIP experiments show that HDACs 1, 5, and 7 are associated with the Rta promoter in latently infected cells, and nuclease mapping shows that a nucleosome is stably positioned over the Rta start site under these conditions (Lu *et al.*, 2003). Treatment with an HDAC inhibitor releases the positioned nucleosome, recruits the Ini1/Sn5 subunit of the BRG1 chromatin remodeling complex, and induces Rta expression (Lu *et al.*, 2003). These data suggest that chromatin remodeling of the Rta promoter, specifically at its start site, may be the molecular mechanism that is critical for a reactivation signal to be effective. The relief of repressive chromatin by recruitment of histone acetyltransferases (HATs) to critical DNA elements of the EBV genome also appears to be a crucial regulatory event (Jenkins *et al.*, 2000).

How are HDACs recruited to the Rta promoter to repress its expression and maintain KSHV latency? Bisulfite genomic sequencing demonstrates that the Rta promoter is highly methylated at CpG dinucleotides during latency; premethylation of the Rta promoter prior to transfection severely represses its activity in transient reporter assays (Chen *et al.*, 2001). These data suggest that the methyl CpG binding protein MeCP2 might bind to methylated bases, and recruit HDAC complexes that promote latency. Indeed, treatment of BCBL-1 cells with 5-azacytidine, a DNA methyltransferase inhibitor, reactivates the virus from latency (Chen *et al.*, 2001). Remarkably, even TPA treatment of BCBL-1 cells seems to relieve the repressive effects of methylation of the Rta promoter (Chen *et al.*, 2001), suggesting that regulation of the methylation status of the promoter might be a general mechanism to regulate the latent-to-lytic transition of KSHV. Concordant with the orchestration of the KSHV lytic cycle by Rta and pathogenic progression, the ORF50/Rta promoter in a latent KSHV carrier was highly methylated, while it was virtually unmethylated in most patients with MCD, PEL, and KS (Chen *et al.*, 2001).

However, there are also examples in which lytic cycle genes can be induced independently of Rta expression.

IFNα treatment of BCP-1 cells directly induces vIL-6 from latent KSHV in an IE fashion in the absence of any other viral gene expression (Chatterjee *et al.*, 2002). Twenty-four KSHV genes are induced by forced over-expression of constitutively active Notch in PEL cells, in the absence of induction of Rta (Chang *et al.*, 2005) or productive reactivation of the virus (Carroll *et al.*, 2006). These data suggest that pathways of lytic gene induction that are independent of Rta expression can develop in latently infected cells.

Lytic replication and interactions with the host cell

G1 arrest

It is postulated that many herpesviruses actively promote G1 arrest to favor efficient lytic replication (for review see Flemington, 2001), a function that appears to have been conserved by KSHV. Induction of the lytic cycle by TPA in JSC-1 or BCP-1 (PEL) cells arrests them in G1, as measured by an increase in the 2N DNA content and lack of BrdU incorporation (Wu *et al.*, 2002; Izumiya *et al.*, 2003b). Stable expression of K8/KbZIP in BCBL-1 cells can further delay G1 progression after TPA induction (Izumiya *et al.*, 2003b). The G1 arrest is reproduced by expression of the K8/KbZIP protein in KSHV-negative cells (Wu *et al.*, 2002), in which K8/KbZIP cooperates with C/EBPα to transcriptionally activate the p21 cyclin-dependent kinase inhibitor WAF1/CIP in a p53-independent manner (Wu *et al.*, 2002). These effects require a direct interaction of both proteins through their respective leucine zippers, and do not occur if K8/KbZIP is expressed in C/EBPα null cells (Wu *et al.*, 2002). K8/KbZIP also regulates both C/EBPα and p21 post-translationally; in in vitro proteosomal degradation assays using BCBL-1 extracts, K8/KbZIP stabilizes both the C/EBPα and p21 proteins in a leucine-zipper dependent manner (Wu *et al.*, 2002). K8/KbZIP can also inhibit the kinase activities of the cdk2/cyclinA and cdk2/cyclin E complexes by directly binding to them through the KbZIP basic domain (Izumiya *et al.*, 2003b). Nonetheless, the K8/KbZIP protein itself is phosphorylated by cdk/cyclinA in complexes immunoprecipitated from induced BCBL-1 cells (Polson *et al.*, 2001).

Expression of a second lytic cycle protein, the vGPCR, also promotes a G1 arrest in reactivating PEL cells (Cannon *et al.*, 2006). vGPCR mediates this effect by inducing expression of the cdk inhibitor protein p21 to block cdk 2 activity. Interestingly, this results in counteracting the TPA-induced expression of viral lytic cycle proteins including the lytic switch Rta, and ORF26.

Apoptosis

Expression of Rta in uninfected BJAB cells results in apoptosis, but stable, tetracycline-regulated induction of Rta in BCBL-1 (PEL) cells does not lead to cell death, even up to 13 days following initial expression. These data suggest that, in the absence of viral infection, Rta is proapoptotic, but that a protein(s) expressed in infected cells counteracts cell death induction (Nishimura *et al.*, 2003). KSHV encodes various lytic cycle proteins that are candidates for inhibiting Rta-induced programmed cell death, and use remarkably different mechanisms. Rta itself can inhibit p53-mediated cell death (Gwack *et al.*, 2001b), and can also function as a ligand-independent inducer of the antiapoptotic STAT3 protein (Gwack *et al.*, 2002). K8/KbZIP binds to p53 to repress its transcriptional activity (Park *et al.*, 2000). KSHV ORF16 encodes vBcl-2, which partially reverses cell death induced by Bax (Cheng *et al.*, 1997; Sarid *et al.*, 1997), and counteracts the ability of the pro-apoptotic protein Diva to inhibit Bcl-XL. The K7/viral inhibitor of apoptosis protein (IAP) is a mitochondrial membrane protein that employs at least three different strategies: (i) it helps target cellular Bcl-2 to activated caspase-3 to inhibit its function (Wang *et al.*, 2002); (ii) it binds a cellular cyclophilin ligand to enhance Ca^{2+} flow and protect cells from mitochrondrial damage (Feng *et al.*, 2002); and (iii) it promotes degradation of IkB and p53 by preventing the cellular PLIC1 protein from protectively binding to them (Feng *et al.*, 2004). K9 encodes vIRF-1, a homologue of cellular IRFs, that inhibits apoptosis induced by various means and is required for reactivation of KSHV and expression of lytic genes (Burysek *et al.*, 1999; Flowers *et al.*, 1998; Gao *et al.*, 1997; Jayachandra *et al.*, 1999; Kirchhoff *et al.*, 2002; Li *et al.*, 1998; Lin *et al.*, 2001; Nakamura *et al.*, 2001; Seo *et al.*, 2001). K9 can inhibit p53-dependent transcription (Seo *et al.*, 2001; Nakamura *et al.*, 2001), and blocks transcriptional activation by the cellular proteins IRF-1 and IRF-3 (Burysek *et al.*, 1999; Flowers *et al.*, 1998; Kirchhoff *et al.*, 2002; Li *et al.*, 1998; Lin *et al.*, 2001).

Shut-off of host gene expression

At early times during reactivation of KSHV (10–12 hours post-induction), global mRNA turnover is accelerated in KSHV infected endothelial cells (Glaunsinger and Ganem, 2004). Overexpression of the KSHV ORF37 gene reproduces this effect, so has been termed the SOX protein (for shut-off and exonuclease). Viral transcripts seem to be largely unaffected by the SOX protein, while only a limited number of host transcripts can escape the shut-off. These host transcripts include those encoding HIF-1α and IL-6, both of which play prominent roles in KSHV pathogenesis.

Regulation of lytic DNA replication

A subset of KSHV delayed–early RNAs encode enzymes required for lytic replication of the viral genome, and regulatory factors that initiate and control the DNA replication process. Viral DNA synthesis begins shortly after the appearance of the delayed–early proteins. As in other herpesviruses, KSHV lytic phase replication proceeds via a mechanism that is distinct from latent viral DNA replication (discussed in detail in the Chapter 24). First, latent DNA replication initiates at *ori-P* and proceeds bidirectionally, while viral lytic replication initiates from a distinct origin (*ori-Lyt*) and proceeds via a rolling-circle mechanism. Second, latent DNA replication depends on host cellular DNA polymerase and accessory factors, while viral lytic replication utilizes a DNA polymerase and other factors encoded by KSHV. Third, latent viral replication occurs in synchrony with the host cell to maintain a stable number of viral episomes per host genome, while viral lytic replication amplifies the viral DNA by one-hundred- or even one-thousand-fold.

Origins of lytic DNA replication

Lytic cycle DNA replication of KSHV initiates from two lytic origins (*ori-Lyt-L and ori-Lyt-R*), located in the genome between K4.2 and K5, and between K12 and ORF71, respectively (AuCoin *et al.*, 2002; Lin *et al.*, 2003). Both *ori-Lyt*s share an almost identical 1.1 kb core sequence, juxtaposed to 600 bp GC-rich repeats (Fig. 26.4).

Each 1.7 kb *ori-Lyt* sequence is necessary and sufficient as a *cis*-acting signal for KSHV lytic replication in transient replication assays (Lin *et al.*, 2003). However, in the context of the viral genome, *ori-Lyt-L* appears to be sufficient to propagate the viral genome whereas *ori-Lyt-R* alone seems inert to direct amplification of viral DNA, as suggested by an investigation using recombinant KSHVs that removed one or both of the *ori-Lyts* in the genome (Xu *et al.*, 2006). Three essential sequence motifs are conserved in the 1.1 kb. region: (i) an 18-bp AT-palindromic sequence, (ii) eight C/EBP binding motifs, arranged as four spaced palindromes, and (iii) an ORF50/Rta responsive element (RRE) linked to a TATA box (Wang *et al.*, 2004b). Substitution or deletion of any of these three core elements abolishes *ori-Lyt* function (Wu *et al.*, 2003; Wang *et al.*, 2004b), while the 600-bp GC-rich tandem repeats are also required for efficient DNA replication (Lin *et al.*, 2003; Wang *et al.*, 2006).

The AT-palindromic sequence (Fig. 26.5) is also present in the core loop structures of HSV-1 *ori-L* and *ori-S,* as well as in the HHV6 and HHV-7 lytic origins. In fact, an AT-rich palindrome is a common feature of both cellular and viral DNA replication origins (Challberg and Kelly, 1989; DePamphilis, 1993). It is believed that an AT-rich palindrome facilitates DNA unwinding and enhances helicase activity during DNA replication.

The eight C/EBP binding motifs are organized as four spaced C/EBP palindromic pairs within a 240-bp sequence (Wu *et al.*, 2003; Wang *et al.*, 2004b). Each palindrome contains two head-to-head CCAAT consensus motifs that are separated by a 13- or 12-bp spacer sequence. One of the KSHV origin binding proteins (OBPs), the K8/KbZIP protein, associates with *ori-Lyt* at the CCAAT motifs (discussed below) (Wang *et al.*, 2004b, 2006).

The RRE/TATA box motif functions as a cis-acting transcriptional promoter whose activity is required for replication from *ori-Lyt*. The KSHV ORF50/Rta protein binds directly to the RRE to activate transcription at *ori-Lyt*. The promoter in *ori-Lyt-L* normally directs transcription of a 1.4 kb RNA containing the GC-rich repeats and a putative open reading frame of 75 amino acids, and the promoter in *ori-Lyt-R* controls the synthesis of the 2.3-kb mRNA encoding K12 (Wang *et al.*, 2004b). Transcription events controlled by these promoters are essential for DNA replication from the *ori-Lyt*, as prematurely termination of the transcription by inserting an SV40 polyadenylation sequence upstream of the GC-rich repeats completely abolished the transcription activity as well as DNA replication (Wang *et al.*, 2006). The EBV *ori-Lyt* also contains a promoter whose transcriptional activity is required for replication (Hammerschmidt and Sugden, 1988), but the role of these promoters and transcripts in gamma-herpesviral lytic replication are unknown.

The 600-bp GC-rich tandem repeat sequences adjacent to the *ori-Lyt* core sequences are represented as both 20-bp and 30-bp tandem arrays in the *ori-lyt-L,* and two types of 23-bp tandem repeats in the *ori-Lyt-R.* Such GC-rich tandem repeats are also found in the *ori-Lyts* of EBV and RRV (Hammerschmidt and Sugden, 1988; Pari *et al.*, 2001).

K8/KbZIP and ORF50/Rta are origin-binding proteins that are responsible for recruiting pre-replication complexes to *ori-Lyt* DNA

Initiation of herpesviral lytic replication requires binding of OBPs to *ori-Lyt* elements. Subsequently, the OBPs recruit DNA replication enzymes and accessory factors to the origins. The recruitment of replication proteins by OBPs is dictated by specific protein–protein interactions, and facilitated by linking and distortion of the origin DNA by the OBPs.

All four of the C/EBP palindromes in *ori-Lyt* are indispensable for replication, and mutagenesis studies have

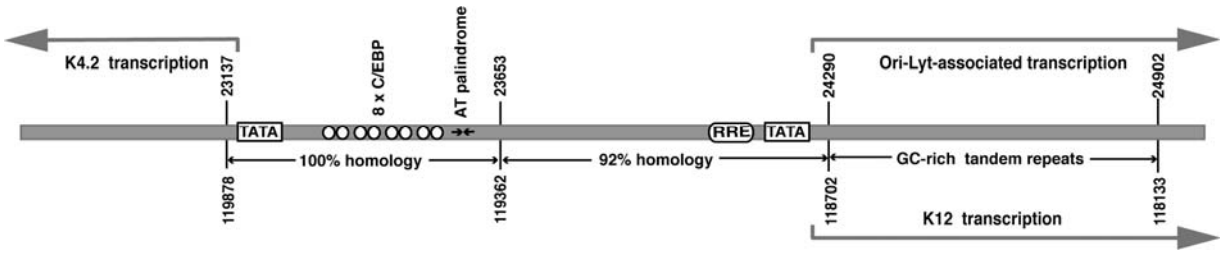

Fig. 26.4. Structure of the KSHV origin of DNA replication (*ori-Lyt*). The positions of various characteristic motifs (TATA boxes, C/EBP binding motifs, AT palindrome, RRE and GC tandem repeats) are as indicated. The homologies of subregions between two *ori-Lyts* are compared and shown on the bottom.

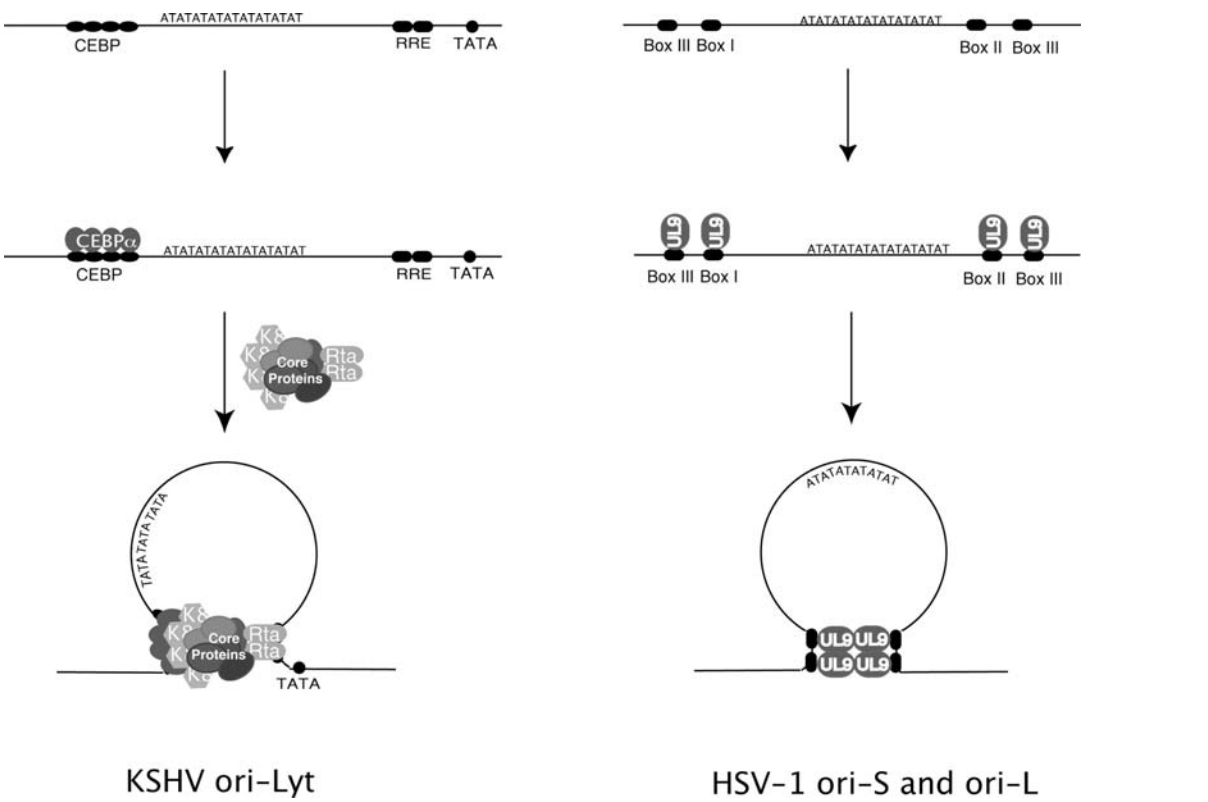

Fig. 26.5. Model for recruitment of core replication proteins to KSHV *ori-Lyt* DNA through ORF50/Rta and K8/KbZIP. Six core replication machinery proteins (referred to as core proteins for simplicity), ORF50/Rta and K8/KbZIP (referred to as Rta and K8, respectively, for simplicity) form a pre-replication complex regardless of the presence of *ori-Lyt* DNA. The pre-replication complex is loaded at a KSHV *ori-Lyt* by a two-point-contact through RTA and K8, each of which interacts with their binding motifs in the *ori-Lyt*. The interaction may lead to looping and distortion of the *ori-Lyt* DNA (left panel), which resembles the binding and looping of HSV-1 *ori-S* and *ori-L* by UL9 proteins (right panel)

shown that three of the individual C/EBP sites C/EBP 1, 2, and 6 are essential for *ori-Lyt* function. Although deletion or mutation of these C/EBP motifs also impairs the association of K8/KbZIP with the *ori-Lyt*, subsequent EMSA experiments have failed to demonstrate direct protein-DNA interactions between the two (Wang *et al.*, 2004b). However, the observation that (i) C/EBP α binds directly to four of the C/EBP sites, and (ii) C/EBP α binds specifically to the K8/KbZIP protein (Wu *et al.*, 2003), implies that K8/KbZIP binds to KSHV *ori-Lyt* through interacting with DNA-bound C/EBP α (Fig. 26.5) (Wang *et al.*, 2004b).

Binding of the ORF50/Rta protein to the RRE in *ori-Lyt* is absolutely essential for *ori-Lyt*-dependent DNA replication, suggesting that Rta is a second critical OBP. The RRE closely resembles the RRE sequence in the nut-1/PAN promoter (Wang *et al.*, 2004b; AuCoin *et al.*, 2004), and deletion of the RRE or the downstream TATA box abolishes transcription and replication at *ori-Lyt*. If the RRE is replaced by a binding site for the heterologous Gal4 protein, a Gal4-ORF50 fusion protein can rescue replication, suggesting that Rta-mediated transactivation is required for replication. In addition to the transcriptional activation Rta also participates in replication complex formation on *ori-Lyt* by interacting with the K8/KbZIP protein and other core replication machinery proteins (Wang *et al.*, 2006). This notion is derived from several lines of evidence as follows: (i) Rta was found to be a component of viral replication compartments in the nuclei of infected cells, and shown to interact with core replication machinery complexes (or pre-replication complexes) composed of at least six core replication proteins and K8/KbZIP, (ii) The loading of the core replication machinery complexes on the *ori-Lyt* DNA appears to be mediated by Rta and to be dependent upon the RRE in the *ori-Lyt*, as the deletion of the RRE abolished the association of replication proteins with *ori-Lyt* DNA. This suggests that Rta plays a role in recruiting the core replication machinery complexes to the *ori-Lyt* DNA, (iii) The association of Rta with pre-replication complexes is not DNA-mediated. Instead, the complexes are assembled independent of the presence of *ori-Lyt* DNA and become less stable once they are recruited and loaded on *ori-Lyt* DNA, perhaps in order to convert to replication fork complexes (Wang *et al.*, 2006).

K8/KbZIP also interacts with core replication machinery proteins and has a similar function as Rta in recruiting and loading pre-replication complexes to the *ori-Lyt* DNA. Thus, formation of a functional DNA replication initiation complex on an *ori-Lyt* requires the efforts of both RTA and K8/KbZIP in loading pre-replication complexes on *ori-Lyt* (Wang *et al.*, 2006). Based on these data, a model of K8/KbZIP and ORF50/Rta recruiting pre-replication complexes to *ori-Lyt* is proposed in Fig. 26.5. In this model, a pre-replication complex apparently binds to an *ori-Lyt* DNA through two contact points, one at the K8 binding region (the C/EBP cluster) near one end of the *ori-Lyt* and the other at the RRE near the opposite end. The two-contact-point binding may bring the two ends of the *ori-Lyt* element together, looping the DNA between the K8 and Rta binding sites. An 18-bp AT palindrome sequence is located in the looped region (Fig 26.5). A similar loop structure has been seen in the HSV-1 DNA replication system, in which two UL9 protein dimers (OBP) bind to two binding sites (Boxes I and II) in *ori-S* and *ori-L* and the protein-protein interactions between the UL9 molecules loops the *ori-Lyt* DNA centered by an AT-palindrome sequence (reviewed in Boehmer and Lehman, 1997). The looping leads to bending and distortion of *ori-Lyt* DNA, facilitating unwinding of the DNA sequence.

Viral enzymes and accessory factors essential for KSHV ori-Lyt-specific DNA replication and DNA replication machinery

Following binding of the OBPs to an origin, DNA replication enzymes and accessory factors are recruited to the origin, and DNA replication begins. Herpesvirus-encoded enzymes and trans-acting factors essential for lytic DNA replication are referred to as the core replication machinery. Homologues of the core proteins have been found in all herpesviruses studied to date, and are structurally and functionally conserved. In fact, both the HSV-1 and KSHV-encoded replication proteins can replicate the EBV *ori-Lyt* in the presence of the EBV OBP *Zta* (Fixman *et al.*, 1995; Wu *et al.*, 2001).

The candidate KSHV replication core machinery proteins were originally identified by sequence similarity to HSV-1 and EBV homologues (see Table 26.1) (Wu *et al.*, 2001; AuCoin *et al.*, 2004), and include (i) a DNA polymerase (POL encoded by ORF9), (ii) a polymerase processivity factor (PPF by ORF59), (iii) a single-stranded DNA binding protein (SSB by ORF6), (iv) a helicase (HEL by ORF44), (v) a primase (PRI by ORF56), and (vi) a primase-associated factor (PAF by ORF40/41). Co-transfection of expression vectors for these proteins and the OBPs (K8/KbZIP and ORF50/Rta) is necessary and sufficient to support DNA replication of a plasmid containing a KSHV *ori-Lyt* in mammalian cells. If any of the core replication proteins are omitted in the system, no replicated KSHV *ori-Lyt* plasmid DNA is detected (AuCoin *et al.*, 2004). In these crucial transient co-transfection-replication assays, replicated *ori-Lyt*-containing DNA becomes resistant to methylation-sensitive restriction enzymes that can only digest the input

Table 26.1. KSHV DNA replication proteins and their analogues in other herpesviruses

			KSHV vs. HSV-1			KSHV vs. EBV			
Protein and enzyme	Gene	Size (aa)	Name	Sim(%)	Iden(%)	Name	Sim(%)	Iden(%)	Activities
K8/KbZIP	K8	237				BZLF1		–	*Ori-Lyt binding protein*
ORF50/Rta	ORF50	691				BRLF1	32.2	20.6	Transcription activator, *ori-Lyt* binding protein
SSB	ORF6	1133	UL29	36.1	27.1	BALF2	65.5	42.1	Single-stranded DNA binding
HEL	ORF44	788	UL5	49.6	37.7	BBLF4	67.8	51.1	Helicase subunit
PRI	ORF56	843	UL52	39.5	29.0	BSLF1	56.6	35.4	Primase subunit
PAF	ORF40/41	670	UL8	—		BBLF2/3	47.1	23.3	Primase-associated factor
POL	ORF9	1012	UL30	60.0	41.7	BALF5	70.9	55.6	DNA polymerase subunit
PPF	ORF59	396	UL42	—		BMRF1	50.7	28.3	Double-stranded DNA binding protein, DNA polymerase processivity factor subunit

aa: amino acid; Sim%: percent similarity; Iden%: percent identity.

plasmid; the replication products are then detected by Southern blotting. The six core replication proteins can form a large globular shaped replication compartment even in the absence of an *ori-Lyt* DNA (Wu *et al.*, 2001), suggesting that they spontaneously assemble a pseudo-replication compartment (without DNA) in solution, then land on with the *ori-Lyt* DNA aid of OBPs. The biochemical properties of the individual KSHV DNA replication proteins are reviewed as follows:

Single-stranded DNA binding protein (SSB)

Among the core replication proteins, SSB is one of two intrinsic nuclear proteins (the other is PPF) that contributes to the nuclear translocation of the other core proteins, especially the primase-helicase tripartite subcomplex proteins PAF, HEL and PRI (Wu *et al.*, 2001; AuCoin *et al.*, 2004). The mechanism of KSHV SSB has not been studied in detail, but is probably similar to that of its HSV-1 counterpart (for review see Boehmer and Lehman, 1997). SSB rapidly and cooperatively binds to single-stranded *ori-Lyt* DNA formed as a result of binding of OBP proteins, and unwinds short regions of duplex DNA to further destabilize the DNA helix. These functions of SSB promote viral DNA replication by enhancing subsequent DNA polymerase and DNA helicase-primase activities.

DNA helicase–primase complex

The KSHV proteins HEL, PRI, and PAF form a tripartite primase/helicase complex, similar to their homologues in HSV-1 and EBV. Unlike its counterparts, efficient translocation of the KSHV complex to the nucleus requires co-expression of the three other core replication proteins (Wu *et al.*, 2001). High homologies between the KSHV and the HSV-1 proteins (or subunits if the tripartite complex is considered a holo-enzyme) suggest similar modes of action. The HSV-1 holoenzyme consists of a 1:1:1 association of UL5 (HEL), UL52 (PRI) and UL8 (PAF) proteins, and has a native molecular mass of 270 kDa. However, the UL5/UL52 sub-assembly displays DNA-dependent ATPase, 5′–3′ helicase, and primase activities, as well as a primer synthesis function that is stimulataed by the UL8 protein (reviewed in Boehmer and Lehman, 1997).

DNA polymerase and processivity factor

As in other herpesviruses, the KSHV DNA polymerase consists of two subunits, POL (polymerase) and PPF (polymerase processivity factor). KSHV POL not only shares a high homology with its HSV-1 counterpart, but also with DNA polymerases from mammals, yeast, *E. coli,* and bacteriophage. In the absence of the PPF subunit, the KSHV POL incorporates only several nucleotides from a primer template. However, association with the PPF enables POL to incorporate thousands of nucleotides continuously without dissociation from the template (Lin *et al.*, 1998).

Association with a processivity factor is an evolutionarily-conserved mechanism by which DNA polymerases achieve catalytic efficiency. The most conserved processivity factors are the ring-shaped sliding clamps, which encircle DNA templates and enable the associated DNA polymerases to slide along the template without dissociating. However, the HSV-1 processivity factor

is of insufficient size to form an encircling sliding clamp; instead, it tethers the DNA polymerase to the DNA template by double stranded DNA binding. The KSHV and HSV-1 PPF subunits display only limited sequence similarity, ~40 amino acids near their carboxy termini, so the mechanism of KSHV PPF remains elusive (Chen *et al.*, 2004). Since processivity factors adopt divergent states during DNA replication, they may serve as good targets for the design of novel and specific anti-herpesviral therapies.

In addition to these essential DNA replication proteins, KSHV also encodes several other enzymes for its DNA replication, including thymidine kinase, ribonucleotide reductase, alkaline exonuclease, uracil N-glycosylase and deoxyuridine triphosphatase. However, the functions of these enzymes are not essential for viral DNA replication.

Late genes and KSHV virion structure

The KSHV virion conserves the major structural elements of herpesviruses, including the genomic DNA, the capsid, the tegument, and the envelope. Proteins required for assembly and structure of DNA viruses have traditionally been regarded as viral late genes that require prior viral DNA replication for expression. This is likely true for the KSHV proteins that comprise the capsid and are inserted into the envelope, although there are exceptions (as discussed above and in Chapter 23). Proteins that comprise the tegument, however, are likely to be expressed from all kinetic classes of lytic replication; as discussed below, the ORF45 protein is an IE protein that is packaged into the KSHV tegument (Zhu and Yuan, 2003).

Virions released from induced PEL cells have provided the first detailed understanding of virion structures for the γ-herpesviruses. When PELs are treated with TPA and sodium butyrate, purified virions contain three types of capsid, named A, B, and C, respectively (Nealon *et al.*, 2001). Fully mature C capsids have a total mass of 300 Mda, and in declining order of abundance, contain the proteins ORF25/MCP (Major capsid protein), ORF65/SCIP (small capsomer-interacting protein), ORF26/TRI-2 (triplex-2), ORF62/TRI-1, and the 160–170 kb (unique region plus repeats) viral genome (Nealon *et al.*, 2001). A capsids contain the four proteins listed above, but lack viral genomic DNA. B capsids, the most abundant category released from lytic PEL cells, have the identical constituents as A capsids but also contain the scaffolding protein encoded by ORF17.5 (Nealon *et al.*, 2001). The capsid components are organized into a discrete series of capsomers containing hexons, pentons, and triplexes of one or two proteins each. The hexons and pentons contain six and five copies of the MCP, respectively, and the triplexes contain a dimer of TRI-2 protein and a monomer of TRI-1 (Trus *et al.*, 2001; Wu *et al.*, 2000). SCIP associates with MCP on hexamers, but not pentamers, and its location at the outermost portions of the capsid suggests that it might directly contact the tegument (Lo *et al.*, 2003). This localization of SCIP to the outer capsid region is conserved in virions of Rhesus monkey rhadinovirus, but not with α-herpesviruses, suggesting a potential unique function for γ-herpesvirus teguments (Yu *et al.*, 2003).

Cryoelectron microscopy at 24-Ångström resolution and digital reconstruction demonstrate that the mature capsid is icosahedral with a diameter of approximately 1140–1300 -Ångström (Wu *et al.*, 2000; Trus *et al.*, 2001). The *T*=16 icosahedral structure of the KSHV capsid is similar to that of the γ-herpesvirus herpes simplex virus-1, and the β-herpesvirus, Human cytomegalovirus, but the overall sizes of each capsid are proportional to the sizes of each virus's genome (Wu *et al.*, 2000; Trus *et al.*, 2001). Phylogenetic comparisons of the MCP protein reveal that the β- and γ-herpesviruses are more closely related to each other than to the α-herpesviruses (Trus *et al.*, 2001).

When protein lysates of purified virions are displayed on SDS-PAGE gradient gels, approximately 30 proteins can be differentiated (Zhu and Yuan, 2003); 24 of these have been identified by proteomic analyses (Zhu *et al.*, 2005). Those that are not capsid proteins are predicted to be parts of the tegument or envelope layers characteristic of mature herpesviral virions, which are presumably added to KSHV capsids during cellular egress. Various glycoproteins of KSHV, including K8.1, glycoprotein (g) B, gH, gL, gM, and gN, have been identified by sequence analysis and/or have been demonstrated to be membrane components of KSHV virions (Akula *et al.*, 2001; Baghian *et al.*, 2000; Pertel, 2002; Birkmann *et al.*, 2001; Wang *et al.*, 2001; Zhu *et al.*, 1999). Detergent treatment of virions releases membrane glycoproteins, but not tegument proteins, which are sandwiched between the capsid and membrane (Zhu *et al.*, 2005). Although the tegument of HSV-1 contains the well-characterized virion protein (VP)16, a transcriptional transactivator essential for expression of HSV-1 IE genes during *de novo* infection (reviewed in (Roizman and Sears, 1996)), the functions of KSHV tegument proteins are virtually unknown. The first tegument protein of KSHV to be identified is the product of ORF45 (Zhu and Yuan, 2003), a protein that counteracts the cellular α-interferon response by blocking the phosphorylation and nuclear accumulation of interferon regulatory factor (IRF)-7 (Zhu *et al.*, 2002). This suggests that ORF45 is packaged into the tegument so that it can function at the earliest stages of *de novo* KSHV infection. Eleven KSHV RNAs are

also found packaged in the virion, and may get translated after infection (Bechtel *et al.*, 2005).

Perspectives

KSHV has conserved the hallmark of herpesvirus pathogenesis: latent infection that persists for the lifetime of the host, requiring successful establishment and maintenance of latency in long-lived or self-perpetuating cellular reservoirs. Selective pressure exerted for millions of years on herpesviruses would not be expected to favor an increase in virulence unless it allowed greater replication and transmission of the virus. The obvious contribution of lytic genes to KS pathogenesis, therefore, is to facilitate dissemination of the virus from its long-term reservoir to sites of disease or of shedding. However, as highlighted in other chapters of this book, twelve viral open reading frames (ORFs) that have independent transforming, pro-angiogenic, pro-growth, anti-apoptotic, or immunomodulatory functions, are expressed following lytic reactivation of the virus. Therefore, it is likely that the pathogenic requirement for KSHV lytic reactivation and replication, as observed in epidemiologic studies, reflects both a necessity for increased viral titers to facilitate viral dissemination, as well as a contribution of viral lytic cycle proteins as direct effectors of the disease phenotypes. Lytically infected cells may thus serve not only as reservoirs of infectious virus but also as reservoirs of pathogenic viral proteins. Translated to the clinical setting, this may represent a pathogenic mechanism by which KSHV initiates or maintains the aggressive inflammatory infiltrate that characterizes KS lesions.

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EBV gene expression and regulation

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Introduction

Epstein–Barr virus (EBV) is an extremely efficient virus infecting the majority of the world's adult population (Rickinson and Kieff, 2001). Following primary infection, EBV persists in the infected host as a lifelong asymptomatic infection. Early in the course of primary infection, EBV infects B-lymphocytes, although it is not known where B-lymphocytes are infected and whether this involves epithelial cells of the upper respiratory tract. To achieve long-term persistence in vivo, EBV colonizes the memory B-cell pool where it establishes latent infection, which is characterized by the expression of a limited subset of virus genes, known as the "latent" genes (Thorley-Lawson, 2001). There are several well-described forms of EBV latency, each of which is utilized by the virus at different stages of the virus life cycle and which are also reflected in the patterns of latency observed in the various EBV-associated malignancies (Rickinson and Kieff, 2001; Young and Murray, 2003). Furthermore, during its life cycle EBV must periodically enter the replicative cycle in order to generate infectious virus for transmission to other susceptible hosts, although it is also not clear whether this occurs in B-lymphocytes or in other cell types of the oropharynx (Rickinson and Kieff, 2001).

This chapter describes the EBV latency and replicative programs utilized by the virus as a means to understand how the virus infects and then establishes persistence in the host. The function of each of the key latent and lytic genes will be described in detail and will provide the foundation for later chapters which describe the contribution of some of these EBV genes to the pathogenesis of the EBV-associated malignancies.

Virus and genome structure

EBV is a gammaherpesvirus of the *Lymphocryptovirus* (LCV) genus and is closely related to other LCVs present in Old World non-human primates, including EBV-like viruses of chimpanzees and rhesus monkeys. In fact, the rhesus monkey LCV and EBV share similar sequences and genetic organization, and are both capable of maintaining infection in the oropharynx and in B cells (Moghaddam *et al.*, 1997). Recently, a transforming, EBV-related virus has also been isolated from spontaneous B cell lymphomas of common marmosets and is thus the first EBV-like virus to be identified in a New World monkey species (Cho *et al.*, 2001; Rivailler *et al.*, 2002). Sequencing of the genome of the marmoset LCV revealed considerable divergence from the genomes of EBV and Old World primate EBV-related viruses.

The EBV genome is composed of linear double-stranded DNA, approximately 172 kilobase pairs (kb) in length. EBV has a series of 0.5 kb terminal direct repeats (TRs) and internal repeat sequences (IRs) that divide the genome into short and long, largely unique sequence domains (Fig. 27.1(a)). The infectious mononucleosis-derived, B95–8 strain of EBV was the first herpesvirus to have its genome completely cloned and sequenced (Baer *et al.*, 1984; Accession number V01555). This prototype B95-8 strain of EBV is atypical of the majority of isolates, in particular it is missing an 11.8 kb segment of the genome (Fig. 27.1). In order to present a sequence more representative of the majority of isolates, a "hybrid" sequence has been assembled (accession number NC_007605) in which the 11.8 kb sequence determined from the Raji strain of EBV (Parker *et al.*, 1990) has been

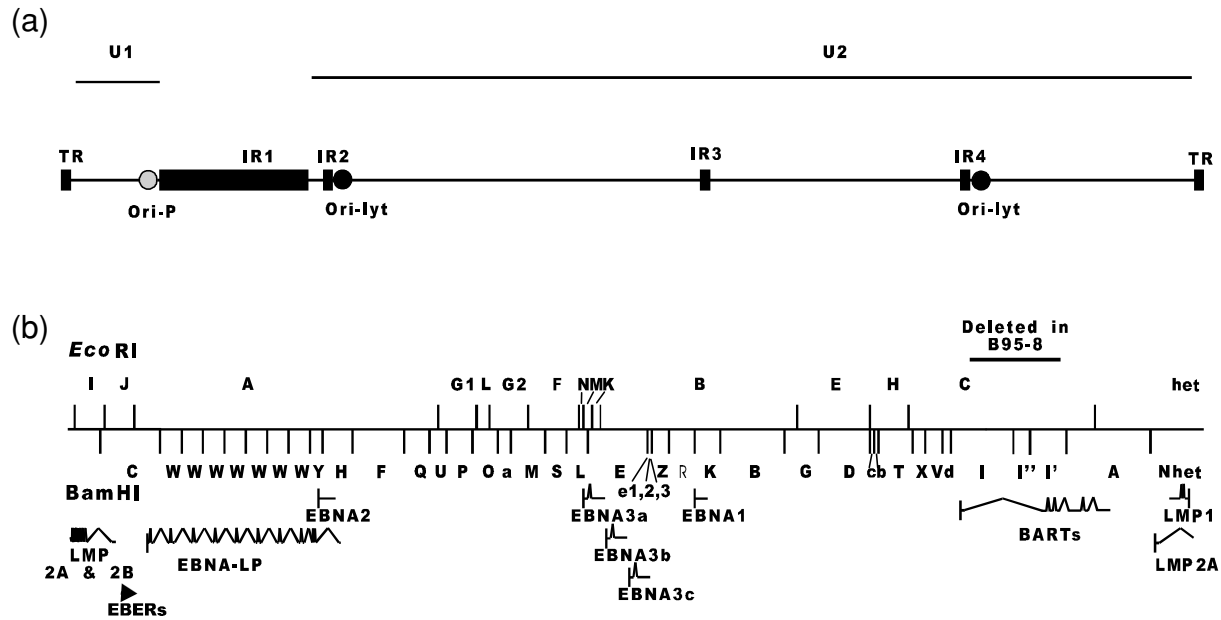

Fig. 27.1. The Epstein–Barr virus (EBV) genome. (a) General organization of linear, EBV virion DNA. U1 and U2 are the short and long unique regions of the genome, respectively. These are interspersed with the four internal repeat regions IR1–4. TR represent the terminal repeats. The origin of replication (Ori-P) of the intracellular, circular, episomal form of the genome is indicated by the grey circle whilst the lytic origins of replication (Ori-lyt) are shown as black circles. (b) *Eco*RI and *Bam*HI restriction endonuclease maps of EBV DNA and the positions of the genes expressed in latency. The *Bam*HI fragments are named according to the well-established B95–8 designations with the exception of I (which is larger than its B95–8 counterpart), I′ and I″ (which are absent in the B95–8 strain). The splicing patterns of the latent RNAs through the coding regions are indicated. EBNA-LP is transcribed from variable numbers of repetitive exons. The full transcriptional patterns of the remaining EBNA genes are more complex than shown here (see text) and the BARTs consist of a number of differently spliced RNA species emanating from the same promoter (Smith *et al.*, 2000; de Jesus *et al.*, 2003). Note that the LMP2 proteins are produced from mRNAs that splice across the terminal repeats in the intracellular, circularised EBV genome. This region has often been referred to as Nhet to denote the heterogeneity in this region according to the number of terminal repeats within different virus isolates.

inserted into the B95–8 sequence to make good the deletion. Recently the complete sequences of two more strains of EBV have been determined: GD1, derived from a Chinese nasopharyngeal carcinoma patient (Zeng *et al.*, 2005; accession number AY961628) and AG876 (Dolan *et al.*, 2006; accession number DQ279927), derived from a Ghanaian Burkitt lymphoma (Pizzo *et al.*, 1978). Since the prototype B95–8 genome was sequenced from an EBV DNA *Bam*HI fragment cloned library, open reading frames (ORFs), genes and sites for transcription or RNA processing are frequently referenced to specific *Bam*HI fragments, from A to Z, in descending order of fragment size (Fig. 27.1(b)). The virus has the coding potential for around 80 proteins, not all of which have been identified or characterized. Characterized gene products are listed in Table 27.1. Analysis of the sequences of GD1 and AG876 together with correction of the B95–8 sequence has led to the identification of four previously unrecognized open reading frames: BVLF1 and BDLF3.5 whose functions are unknown, BFRF1A which by homology with other Herpesviruses probably has a role in DNA packaging, and BGLF3.5 which is likely to be a tegument protein (Dolan *et al.*, 2006).

EBV latency in vitro and in vivo

The lymphoblastoid cell line LCL

When peripheral blood lymphocytes from healthy EBV sero-positives are placed in culture, the few EBV-infected B-lymphocytes that are present regularly give rise to spontaneous outgrowth of EBV-transformed, immortalized cell lines, known as lymphoblastoid cell lines (LCLs), provided that immune T-lymphocytes are either removed or inhibited by the addition of cyclosporin A to the culture (Rickinson *et al.*, 1984). LCLs can also be generated by direct infection of resting B-lymphocytes with EBV derived from producer B-cell lines.

Table 27.1. List of characterised genes. References pertain to genes that are not considered further in the text. Open reading frames within the EBV genome are named sequentially after the *Bam*HI fragment within which they begin and the direction in which they are read. Thus, for example, BZLF1 refers to ***B****am*HI-**Z** **l**eftward reading **f**rame number **1**.

Latent genes		
ORF	***Gene product***	**Reference**
BKRF1	EBNA1	
BYRF1	EBNA2	
BLRF3/BERF1	EBNA3A (EBNA3)	
BERF2a/b	EBNA3B (EBNA4)	
BERF3/4	EBNA3C (EBNA6)	
*Bam*HI-W (repeated multiple splices)	EBNA-LP (EBNA5)	
BNLF1	LMP1	
Fused TRs (multiple splices)	LMP2A/B	
BARTs	A73, RPMS1	
EBER1/2	small RNAs	
Early genes		
BZLF1	ZEBRA/Zta/EB1	
BRLF1	Rta	
BRRF1	transcription factor	
BORF2	ribonucleotide reductase large subunit	
BaRF1	ribonucleotide reductase small subunit	
BXLF1	thymidine kinase	
BGLF5	alkaline exonuclease	
BLLF3	dUTPase	Sommer *et al.*, 1996
BKRF3	uracil DNA glycosylase	Olsen *et al.*, 1989
BALF5	DNA polymerase	
BMRF1	polymerase accessory protein	
BALF2	DNA binding protein	
BSLF1	primase	
BBLF2/3	primase accessory protein	
BBLF4	helicase	
BMLF1	mRNA export factor	
BSLF2	spliced to BMLF1	Cook *et al.*, 1994
BHRF1	bcl-2 homologue	
BALF1	viral-bcl-2 antagonist	
BARF1	transformation-associated?	
BGLF4	protein kinase	Chen *et al.*, 2000; Kato *et al.*, 2001
BFRF1	37kDa membrane/virion protein	Farina *et al.*, 2000
BHLF1	ss-DNA binding protein	Nuebling and Mueller-Lantzsch, 1989
BHLF2	envelopment protein	Gonnella et al., 2005
BNLF2a	immune evasion	
Late genes		
BNRF1	major tegument protein p143	
BPLF1	large tegument protein	
BOLF1	tegument protein	
BVRF1	tegument protein	
BBLF1	tegument protein	
BGLF1	tegument protein	
BSRF1	tegument protein	
BRRF2	tegument protein	
BDLF2	tegument protein	
BKRF4	tegument protein	
BcLF1	major capsid protein	
BDLF1	minor capsid protein	
BFRF3	capsid protein p18	
BLRF2	capsid protein p23	
BdRF1	capsid protein p40	
BBRF1	capsid protein	
BVRF2	protease	
BGLF2	38Kd protein	
BORF1	may be needed for capsid assembly	Pertuiset *et al.*, 1989
BLRF1	glycoprotein gN	
BLLF1	glycoprotein gp350/220	
BZLF2	glycoprotein gp42	
BKRF2	glycoprotein gL (gp25)	
BBRF3	glycoprotein gM	
BXLF2	glycoprotein gH (gp85)	
BILF1	glycoprotein gp60	
BILF2	glycoprotein gp78/55	
BALF4	glycoprotein B (gp110)	
BDLF3	glycoprotein gp150	
BMRF2	53/55Kd membrane protein	
BALF3	glycoprotein transport?	Pellett *et al.*, 1986
BCRF1	viral IL-10	

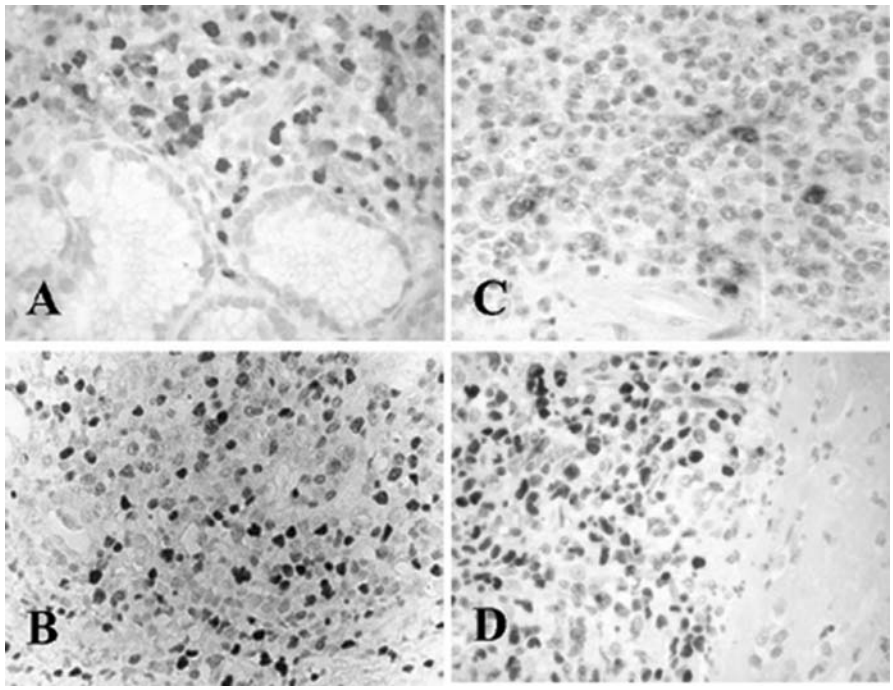

Fig. 27.2. Latency III pattern characteristic of the majority of cases of post-transplant lymphoproliferative disease. All known EBV latent genes are expressed in this form of latency: (a) EBERs, (b) EBNA1, (c) LMP1, (d) EBNA2. (See color plate section.)

Every cell in an LCL carries multiple copies of circular extra-chromosomal viral DNA (episomes) and produces a number of latent proteins, including six nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and -LP) and three latent membrane proteins (LMPs 1, 2A and 2B) (Fig. 27.1(b)). Transcripts, referred to as BARTs (BamHI A rightward transcripts), from the *Bam*HI A region (Bam A) of the viral genome are also detected, although whether these encode proteins remains controversial (Kieff and Rickinson, 2001). In addition to the latent proteins, LCLs also show abundant expression of the small non-polyadenylated RNAs, EBERs 1 and 2; the function of these transcripts is not clear but they are believed to be expressed in all forms of latent EBV infection and have served as excellent targets to detect EBV in tumors (see later chapters). The relative positions and orientations of these viral genes are illustrated in (Fig. 27.1(b)) under a linearized restriction map of the viral genome. The different EBNAs are encoded by individual mRNAs generated by differential splicing of the same long (over 100 kb) "rightward" primary transcript expressed from one of two promotors (Cp or Wp) located close together in the *Bam*HI C and W region of the genome (Speck and Strominger, 1989). A switch from Wp to Cp occurs early in B cell infection as a consequence of the transactivating effects of both EBNA1 and EBNA2 on Cp. The LMP transcripts are expressed from separate promoters in the *Bam*HI N region of the EBV genome, with the leftward LMP1 and rightward LMP2B mRNAs apparently controlled by the same bidirectional promoter sequence (ED-L1) which also responds to transactivation by EBNA2 (Hofelmayr *et al.*, 1999; Kieff and Rickinson, 2001; Speck and Strominger, 1989). The LMP2A promoter is also regulated by EBNA2. Both LMP2A and LMP2B transcripts cross the TRs into the U1 region thus requiring the circularization of the genome for transcription. Circularization occurs by homologous recombination of the TRs resulting in fused termini of unique length and this has been used as a measure of EBV clonality on the assumption that fused TRs with an identical number of repeats denote expansions of a single infected progenitor cell (Raab-Traub and Flynn, 1986). This contention has recently been challenged by the observation that EBV clonality post-infection may be a consequence of the selective growth advantage achieved by optimal LMP2A expression over a minimal number of TRs (Moody *et al.*, 2003). The pattern of latent EBV gene expression observed in LCLs is referred to as the "latency III" (Lat III) form of EBV infection and is characteristic of the majority of post-transplant lymphomas (Fig. 27.2).

The Lat III pattern of EBV gene expression seen in LCLs is matched by an equally consistent and characteristic cellular phenotype with high-level expression of the B-cell

activation markers CD23, CD30, CD39 and CD70 and of the cellular adhesion molecules, leukocyte function associated molecule-1 (LFA-1; CD11a/18), LFA-3 (CD58) and intercellular adhesion molecule-1 (ICAM-1; CD54) (Rowe *et al.*, 1987). These markers are usually absent or expressed at low levels on resting B cells, but are transiently induced to high levels when these cells are activated into short-term growth by antigenic or mitogenic stimulation, suggesting that EBV-induced immortalization can be elicited through the constitutive activation of the same cellular pathways that drive physiological B cell proliferation. The ability of EBNA2, EBNA3C and LMP1 to induce LCL-like phenotypic changes when expressed individually in human B cell lines implicates these viral proteins as key effectors of the immortalisation process (Kieff and Rickinson, 2001; Rickinson and Kieff, 2001; Wang *et al.*, 1990).

Other forms of EBV latency

The examination of EBV latent gene expression in virus-associated tumors and in cell lines derived from Burkitt lymphoma (BL) biopsies identified at least two additional forms of EBV latency. EBNA1 is the only EBV protein consistently observed in EBV-positive BL tumors along with the EBER and *Bam*HIA transcripts; this form of latency is referred to latency I or Lat I (Fig. 27.3) (Gregory *et al.*, 1990; Rickinson and Kieff, 2001; Rowe *et al.*, 1987). Some reports have documented expression of LMP1 and EBNA2 in small numbers of cells in a few cases of endemic BL (Niedobitek *et al.*, 1995), and LMP1 in several cases of sporadic BL (Carbone *et al.*, 1995). In BL biopsies and representative BL cell lines EBNA1 is transcribed from the Qp promoter rather than Wp or Cp (Nonkwelo *et al.*, 1996). Qp, a TATA-less promoter, has many features of the promoters driving expression of housekeeping genes and a downstream element, the Q locus, binds EBNA1 resulting in the repression of Qp transcription (Sample *et al.*, 1992). Recent studies indicate that Qp is also positively regulated by the JAK/STAT pathway (Chen *et al.*, 1999).

BL cells exhibit high level expression of CD10 and CD77, a phenotype most closely resembling that of centroblasts in germinal centers. When cells from some EBV-positive BL tumors are passaged in culture, the other EBNAs and LMPs are expressed, and the EBNA2and LMP1-induced cell surface antigens, such as CD23, CD30, CD39, LFA1, LFA3, and ICAM1, also are up-regulated (Gregory *et al.*, 1990). EBNA2 and LMP1 are the major mediators of EBV-induced B lymphocyte growth in vitro and the lack of expression of these proteins in tumor cells suggests that they are not required for BL growth (Rickinson and Kieff, 2001).

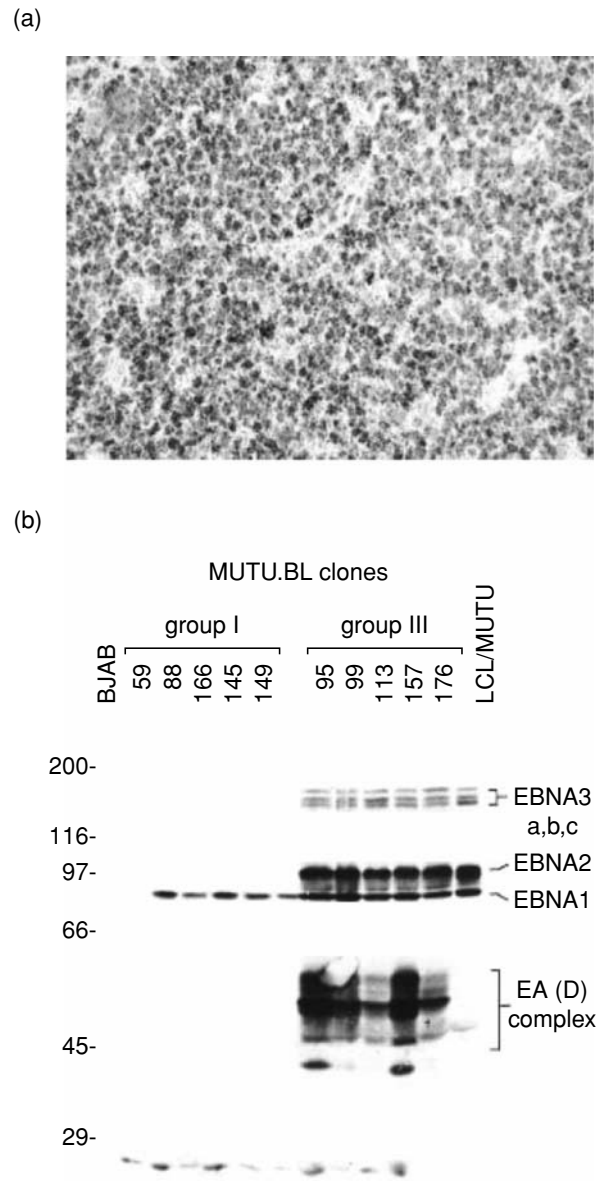

Fig. 27.3. Latency pattern characteristic of Burkitt's lymphoma (BL). (a) Expression of EBERs in the tumor cells of BL tissue shown by isotopic *in* situ hybridization and (b) Immunoblotting demonstrating protein expression limited to EBNA1 in so-called 'group I' BL cell lines which recapitulate the in vivo expression profile. 'Group III' BL lines have an expression pattern similar to that of LCLs (Latency III) and represent cell lines that have 'drifted' from a latency I pattern in vitro. (See color plate section.)

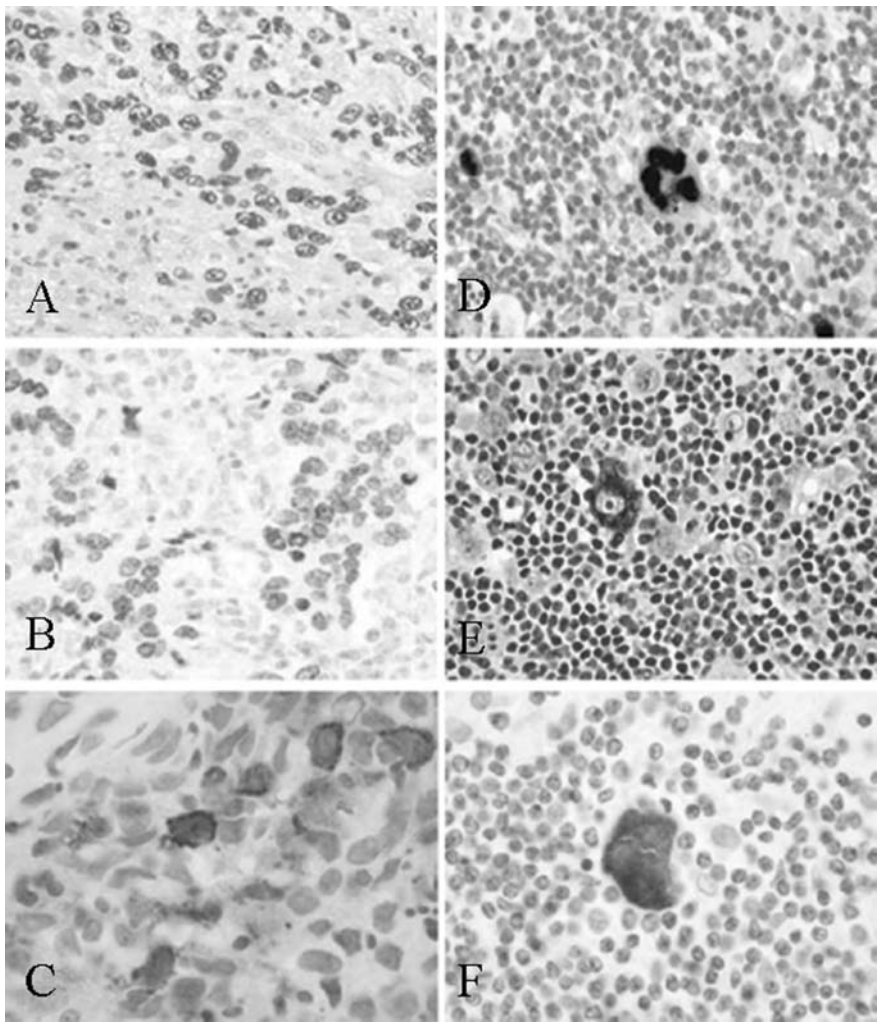

Fig. 27.4. EBV latency type II. Left panel shows (a) Expression of EBERs, (b) EBNA1 and (c) LMP1, in the tumor cells of nasopharyngeal carcinoma (NPC). LMP1 expression is not a regular feature of these tumors. LMP2 protein has not yet been reported in NPC tumors, despite the detection of LMP2 RNA. Right panel shows EBV gene expression in the rare tumor cells of (HRS cells) Hodgkin's lymphoma. (d) EBERs, (e) LMP1 and (f) LMP2. EBNA1 protein is also detectable in the majority of cases (not shown). In contrast to NPC, both LMP1 and LMP2 protein are almost always detectable in EBV-infected HRS cells. (See color plate section.)

Another form of EBV latency, Lat II, was originally identified in biopsies of nasopharyngeal carcinoma (NPC) and subsequently found in cases of EBV-associated Hodgkin's lymphoma (HL) (Brooks *et al.*, 1992; Deacon *et al.*, 1993; Pallesen *et al.*, 1991; Young *et al.*, 1988). Here, expression of the EBERs, Qp-driven EBNA1 and *Bam*HI A transcripts is accompanied by expression of LMP1 and LMP2A/B (Fig. 27.4). Transcription of LMP1 is controlled by an EBNA2-independent promoter located within the viral terminal repeats (L1-TR) and is regulated by the JAK/STAT signaling pathway (Chen *et al.*, 2001, 2003). The factors responsible for LMP2A and LMP2B expression in the absence of EBNA2 in NPC and HL have yet to be defined. Whilst this Lat II pattern of EBV latent gene expression is a consistent feature of virus-associated HL, LMP1 expression in NPC is variable with only around 20% of biopsies being unequivocally positive for LMP1 at the protein level (Niedobitek *et al.*, 1992). The mechanisms underlying differential LMP1 expression in NPC and the consequent effects on the NPC phenotype remain unknown.

The observation that the majority of NPC tumors do not express the Lat II form of EBV latent infection and that EBNA2 can occasionally be detected in BL tumour cells emphasizes the limitations of the operational

categorization of virus latency into three distinct forms. It is clear that in vivo there is often a spectrum of EBV latent and lytic gene expression within the same infected tissue. The need for caution in the rigid application of these forms of EBV latency is highlighted by a recent study in which expression of the EBNA3 family through Wp-driven transcription has been identified in a subset of BL biopsies (Kelly *et al.*, 2002). It appears that in these tumors the selective pressure to down-regulate EBNA2 expression has occurred via deletion of the EBNA2 gene rather than through the switch to Qp usage observed in the conventional BL scenario. Other forms of EBV latency in which the EBERs are not expressed have been reported in breast carcinoma and hepatocellular carcinoma but here the association of EBV with these tumors remains controversial (Bonnet *et al.*, 1999; Murray *et al.*, 2003; Sugawara *et al.*, 1999).

EBV replication/the lytic cascade

The cascade of events in the lytic phase of the EBV life cycle is divided into three phases of regulated gene expression: immediate–early, early and late. The immediate–early gene products are transactivator proteins that trigger the expression of the early genes, the products of which include enzymes that are required for viral DNA replication. In turn, amplification of EBV DNA defines the boundary between early and late gene expression. During the late phase of the cycle viral structural proteins are expressed and assembled into virus particles into which the DNA is packaged prior to release of infectious virions.

The principal switch from latency to productive infection involves activation of the immediate–early genes BZLF1 and BRLF1 (Biggin *et al.*, 1987; Countryman and Miller, 1985; Hardwick *et al.*, 1988; Rooney *et al.*, 1989). On induction of the lytic cycle these two genes are expressed simultaneously (Sinclair *et al.*, 1991) and respectively encode the transactivator proteins known as ZEBRA (Countryman *et al.*, 1987), EB1 (Chevallier-Greco *et al.*, 1986) or Zta (Lieberman *et al.*, 1990) (BZLF1) and Rta (BRLF1). The two proteins can be expressed from a major 2.9 kb and a minor 3.8 kb bicistronic R-Z RNA transcribed from the R-promoter (Rp) whilst ZEBRA is also expressed from a smaller 0.9 kb mRNA initiated at the downstream Z-promoter (Zp). In addition, another 0.9kb message encoding a putative fusion protein known as RAZ and consisting of parts of both Rta and ZEBRA is transcribed at low level (Fig. 27.5) (Manet *et al.*, 1989). The in vivo significance, if any, of the putative RAZ protein is unclear although it appears to be able to act as an inhibitor of ZEBRA (Furnari *et al.*, 1994; Segouffin *et al.*, 1996). Both Zp and Rp are activated by ZEBRA whilst Rta can up-regulate Zp and autoactivate its own synthesis. However, maximum activation of the upstream Rp promoter for the bicistronic messenger is obtained by the synergistic effects of ZEBRA and Rta (Liu and Speck, 2003). This synergism suggests that low levels of the two proteins are sufficient to trigger the lytic cascade. Transcription of the immediate–early genes does not require *de novo* protein synthesis (Biggin *et al.*, 1987) implying that physiological signals from the host cell are involved in triggering activation of the lytic cycle. Lytic inducers activate intracellular signaling pathways that culminate in the dephosphorylation of the Zp-binding myocyte enhancer factor 2D (MEF-2D, see Fig. 27.5) which recruits histone acetylase leading to hyperacetylation of histones within the chromatin structure of Zp and promoter activation (Bryant and Farrell, 2002; Deng *et al.*, 2003; Gruffat *et al.*, 2002b; Jenkins *et al.*, 2000; Speck *et al.*, 1997). It has recently been shown that the BRRF1 gene product is a transcription factor that activates Zp and also cooperate with Rta to induce lytic infection (Hong *et al.*, 2004).

ZEBRA is a member of the basic-zipper family of transcription factors and binds as a homodimer to ZEBRA response elements (ZREs) within early gene promoters. Whilst ZEBRA alone activates some early genes, others are maximally stimulated by the synergistic effects of ZEBRA and Rta, whereas a third class is maximally induced by Rta alone (Feederle *et al.*, 2000; Ragoczy and Miller, 1999).

In common with other herpesviruses, EBV encodes several early genes that are involved in nucleotide metabolism e.g. thymidine kinase (BXLF1) and in DNA replication, e.g., DNA polymerase. Studies of herpes simplex virus (HSV) suggest that many genes of the former class are non-essential for replication of the virus, at least in cell culture, since their functions are duplicated by host cell enzymes (Roizman and Knipe, 2001). Conversely six of the latter, BMRF1 (polymerase-associated factor), BALF2 (single-stranded DNA binding protein), BALF5 (DNA polymerase), BSLF1 (primase), BBLF4 (helicase) and BBLF2/3 (primase accessory protein) have been referred to as core replication genes (Fixman *et al.*, 1992) and are obligatory for viral DNA replication and the progression from the early to late phase of the life cycle.

The lytic origin of replication that is employed in the synthesis of virion DNA, ori-lyt (Hammerschmidt and Sugden, 1988), is distinct from the plasmid DNA replication origin, ori-P, that is used to maintain the episomal virus DNA in synchrony with host cell division during latency. Ori-lyt lies within the *Bam*HI H region of EBV DNA and contains two essential cis-acting regions: the BHLF1 promoter and a second region about 0.5 kb distant that is required for

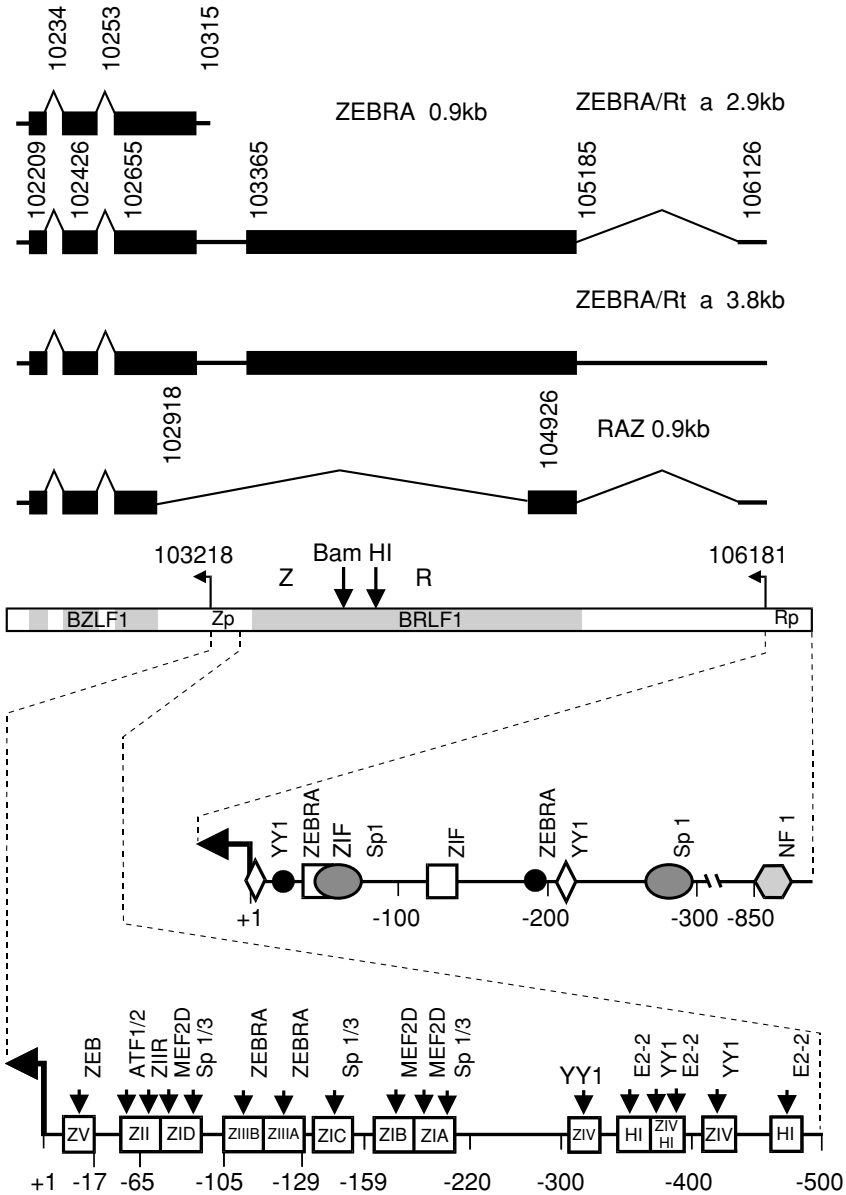

Fig. 27.5. Schematic organization of the immediate–early transactivator genes. The open box in the center represents the BamHI Z-R region of EBV DNA. The BZLF1 and BRLF1 open reading frames and their respective promoters Zp and Rp are indicated with their transcriptional initiation points represented by raised arrows. The four immediate–early transcripts are shown at the top. Horizontal lines represent exons with the coding regions indicated by thicker portions. Genome coordinates are numbered with reference to the B95–8 sequence (Genbank accession number V01555). The organization of the Rp and Zp promoters is illustrated below: +1 refers to the site of transcription initiation. Although Rp contains two ZREs the proximal site is dominant in mediating transcriptional activation by ZEBRA (Liu and Speck, 2003). The NF1 site appears to be a positive regulator in epithelial but not lymphoid cells (Glaser *et al.*, 1998) whilst the Sp1 sites may be involved in the autoactivation of Rp by Rta (Ragoczy and Miller, 2001). The Zif site is a positive trancriptional regulator (Zalani *et al.*, 1995) whereas the YY1 sites are negative regulatory elements that may be involved in the maintenance of latency (Zalani *et al.*, 1997). The Zp promoter (bottom) has been shown to contain multiple regulatory domains (for review see Speck *et al.*, 1997) indicated by the open boxes. Transcription factors that bind to the various domains are marked by the down arrows. The four ZI domains (ZIA – D) are A+T-rich sequences that bind MEF2 transcription factors, principally MEF2D, and seem to play a critical role in the latent to lytic switch (see text). With the exception of ZIB, the ZI domains also bind Sp1 and Sp3 factors. The ZII domain is also potentially responsive to extracellular stimuli by virtue of its affinity for cAMP response element binding (CREB) factors, e.g., ATF1 and 2. In addition, the ZII domain contains a *cis*-acting negative regulatory element (ZIIR) whose putative cellular binding-factor remains unknown (Liu *et al.*, 1998). The ZIII domains bind ZEBRA and are likely involved in autoactivation. The ZIV, ZV and HI domains are negative regulatory elements that bind YY1, zinc finger E-box binding factor (ZEB) and E2–2, respectively (Montalvo *et al.*, 1995; Kraus *et al.*, 2003; Thomas *et al.*, 2003).

replication but not needed for BHLF1 transcription. The BHLF1 promoter contains ZREs that, if mutated, eliminate ori-lyt-directed replication (Schepers *et al.*, 1993). These and other data have demonstrated that ZEBRA is an essential component of lytic DNA replication and it has recently been shown that physical interaction with the EBV helicase targets ZEBRA to viral DNA replication compartments within the nucleus (Liao *et al.*, 2001).

Several herpesviruses replicate their DNA only in G1-arrested host cells (Flemington, 2001); a situation that has been suggested as being advantageous to the virus due to lack of competition with cellular DNA replication. Again ZEBRA has been implicated as a key regulatory element in the EBV lytic cycle. In this instance ZEBRA interacts with C/EBPa leading to accumulation of $p21^{CIP-1}$ and G1 cell cycle arrest (Wu *et al.*, 2003). Further studies suggest that whilst inhibiting cellular DNA synthesis, EBV induces an S-phase-like cellular environment during lytic replication (Kudoh *et al.*, 2003, 2004).

The checkpoint between early and late gene expression involves productive viral DNA replication. How is this linked to the induction of late gene expression? Methylation of promoter sequences can lead to gene repression (Jansson *et al.*, 1992) and thus newly replicated, unmethylated promoter sequences may be activated. However, this mechanism does not appear to be involved since inhibitors of DNA methyltransferase do not activate EBV late genes (Fronko *et al.*, 1989; Szyf *et al.*, 1985). A transient transfection-based reporter assay using EBV early and late promoters within non-replicating plasmids faithfully reproduced the early/late promoter expression profile during different phases of the virus life cycle (Serio *et al.*, 1997). This result demonstrates that a *trans*-relationship exists between viral DNA replication and late gene expression. The steps in the chain of events leading to the activation of late promoters remain to be elucidated. However, Serio *et al.* (1998) provide evidence that a variant TATA element within the core promoter region of the late genes is located within a transcriptionally inert environment and is a critical effector in their regulation. In addition, *cis*-regulatory elements appear to operate in the immediate vicinity of this core to activate late gene expression.

Functions and associated properties of lytic cycle gene products

Lytic cycle gene products were originally identified immunologically as distinct fluorescent antigen complexes referred to as virus capsid antigen (VCA), early antigen (EA) and membrane antigen (MA) (Ernberg and Klein, 1979). These descriptions survive to this day but we now know that these antigens consist of a number of viral structural proteins, replicative enzymes and membrane proteins, respectively. Examination of the sequence of the EBV genome reveals a coding potential for around 80 proteins. Our knowledge of the properties of these gene products encompasses a spectrum of characterization ranging from genes that have been analyzed in minute detail to those that still remain hypothetical. Some aspects of the properties of lytic gene products are summarized below.

Early gene products, early antigens, and diagnostic tests

The early proteins constitute the serologically defined EA complex. More than 20 years ago Henle and colleagues showed that IgA antibodies to EA in the sera of NPC patients were indicative of the disease (Henle *et al.*, 1970). This work was extended as a potential diagnostic and prognostic test for NPC (Zeng *et al.*, 1983) and individual early gene products have now been identified as antigenic determinants and proposed as being potentially useful as more objective diagnostics (Baylis *et al.*, 1989; Connolly *et al.*, 2001; Dardari *et al.*, 2000; Feng *et al.*, 2001; Fones-Tan *et al.*, 1994; Liu *et al.*, 1994; Stolzenberg *et al.*, 1996). More recently, combination assays using more than one target antigen have been evaluated (Chan *et al.*, 2003; Cheng *et al.*, 2002). Whilst several assays appear promising, none has yet been routinely adopted.

DNA replicative enzymes

The six "core replication proteins" together with ZEBRA are absolutely required for the amplification and replication of the viral genome from ori-lyt. The intermediate replication product is a concatemeric structure consisting of several genome units linked head to tail (Hammerschmidt and Sugden, 1988). The enzymatic mechanism involved in cleavage of the concatemers is uncertain (Zimmermann and Hammerschmidt, 1995). The replication proteins have been shown to function as (a) complex(es). The helicase, primase and primase accessory protein physically associate as a nuclear complex (Yokoyama *et al.*, 1999). This heterotrimeric complex interacts (a) with ZEBRA via a helicase association (Gao *et al.*, 1998; Liao *et al.*, 2001) and (b) with the DNA polymerase (Fujii *et al.*, 2000) which itself binds the polymerase accessory protein (Zeng *et al.*, 1997). The DNA binding protein can interact with this tetrameric complex (Gao *et al.*, 1998) and also significantly stimulates DNA synthesis by the core polymerase suggesting an association between these molecules (Tsurumi *et al.*, 1996). These and

other data suggest the following partial scenario for EBV lytic cycle DNA replication: ZEBRA interacts with the helicase/primase/accessory protein complex and recruits this to the ZEBRA binding sites on ori-lyt to form a pre-priming complex to which the DNA binding protein attaches. This complex opens the DNA helix at the origin and synthesizes RNA primers. The DNA polymerase and its accessory protein subsequently attach to the complex allowing DNA replication to proceed (Fujii *et al.*, 2000).

In addition, the ori-lyt-binding host cell encoded transcription factors ZBP-89 and Sp1 interact with the polymerase/accessory protein complex and stimulate viral DNA replication (Baumann *et al.*, 2000). These data suggest an additional, complementary mechanism whereby cellular transcription factors tether the viral replication proteins to ori-lyt.

The mRNA export factor, BMLF1

The early gene product from the BMLF1 ORF is referred to as EB2 (Chevallier-Greco *et al.*, 1986), Mta (Fixman *et al.*, 1992) or SM (Cook *et al.*, 1994). It was originally thought to be a transcriptional transactivator that in conjunction with ZEBRA and Rta regulates lytic cycle activation. It is a heterogeneous, nuclear phosphoprotein with a major species of about 60 kDa.

The export of mRNA from the nucleus to the cytoplasm is significantly enhanced by splicing and requires the action of a multiprotein exon junction complex. However, since the majority of EBV lytic cycle mRNAs are unspliced, an alternative export mechanism such as an mRNA-bound adaptor protein needs to be invoked. The properties of the BMLF1 product are consistent with such a role since it shuttles between nucleus and cytoplasm, contains two nuclear export signals, is an RNA-binding protein in vivo and promotes the cytoplasmic accumulation of unspliced EBV mRNA, notably including some of the core replication genes (Gruffat *et al.*, 2002a; Semmes *et al.*, 1998).

Using a mutant EBV deleted for BMLF1 Gruffat *et al.*, 2002a) showed that EB2/Mta is essential for virion production. The HSV protein homologue of EB2/Mta, ICP27, has been shown to be a viral adaptor molecule that is involved in the nuclear export of intronless viral mRNA (Koffa *et al.*, 2001). ICP27 can partially complement the defect in the BMLF1negative EBV (Gruffat *et al.*, 2002a).

Bcl-2-related proteins

Different members of the bcl-2 family of proteins either induce or repress apoptosis. EBV encodes two bcl-2 homologues; BHRF1 and BALF1. The BHLF1 gene product is a 17 kDa putative membrane protein that is highly conserved in all EBV isolates (Khanim *et al.*, 1997) and is non-essential for virus replication or virus-mediated cellular transformation in vitro (Lee and Yates, 1992; Marchini *et al.*, 1991).

Expression of BHRF1 in a number of cell types in vitro enhances their apoptosis resistance to a variety of appropriate stimuli (Henderson *et al.*, 1993; Kawanishi, 1997; Tarodi *et al.*, 1994). In addition to its anti-apoptotic function BHRF1 promotes rapid transit through the cell cycle (Dawson *et al.*, 1998). These and other data suggest that *in vivo* the primary role of BHRF1 may be to delay host cell apoptosis during the EBV lytic cycle thereby facilitating complete virus replication and assembly (Dawson *et al.*, 1995, 1998).

The second bcl-2 homologue, BALF1, is a 182 amino-acid polypeptide that has counterparts in the EBV-related viruses of other primates (Bellows *et al.*, 2002). This protein appears to act as an antagonist of the anti-apoptotic effects of BHRF1; a situation reminiscent of cellular bcl-2 family members that can counteract each other's functions. How these two proteins interact in the virus's life cycle remains to be determined.

BNLF2a

The $CD8^+$ T-cell response to EBV lytic cycle antigens is markedly skewed towards immediate-early and early proteins whilst late proteins are recognised only rarely (Pudney *et al.*, 2005). Nevertheless, in vivo EBV replicates successfully in spite of this robust immune reponse to lytic antigens. Ressing *et al.* (2005) demonstrated that cells undergoing lytic infection have reduced TAP-mediated transport of peptides to the endoplasmic reticulum and impaired presentation at the cell surface. It has recently been shown that the BNLF2a gene product is the mediator of this immune evasion mechanism by blocking the interaction of peptides with the TAP transporter and likely explains the bias of immune response towards early proteins, i.e. prior to BNLF2a function (A. D. Hislop, personal communication).

BARF1

The BARF1 ORF encodes a product of about 33 kDa that is secreted into the medium of cultured cells (Strockbine *et al.*, 1998). It may be post-translationally modified by N-linked glycosylation, myristylation and phosphorylation (Sheng *et al.*, 2001). It binds to and inhibits the proliferative effects of human colony-stimulating factor 1 (CSF-1). It was proposed that by blocking CSF-1 activity the BARF1 protein might impair cytokine release from mononuclear cells thereby modulating host immune responses to EBV infection (Cohen and Lekstrom, 1999; Strockbine *et al.*,

1998). In addition, a BARF1-negative EBV has revealed a second effect of the BARF1 protein on innate immunity; the deletant was impaired in its ability to inhibit α-interferon production by mononuclear cells (Cohen and Lekstrom, 1999).

Although BARF1 appears temporally as an early gene in the viral life cycle it can act as an oncogene when stably expressed in cultured murine, human or simian cells (Wei *et al.*, 1994, 1997; Wei and Ooka, 1989). In addition, Akata BL cells that had lost their tumorigenic phenotype through loss of the EBV genome regained the ability to form tumors in SCID mice following transfection with BARF1 (Sheng *et al.*, 2003). The N-terminal 49 a.a. appear to be essential for the transforming ability (Sheng *et al.*, 2001). However, a BARF1deleted virus was not defective in its capacity to immortalize B cells in vitro indicating that in the context of the whole virus BARF1 is not essential for this function (Cohen and Lekstrom, 1999).

Nevertheless, observations of the EBV-positive epithelial tumors NPC and gastric carcinoma are provocative. Analysis of BARF1 transcription using RT-PCR or of protein expression by Western blot and immunohistochemistry detected the presence of this antigen in around 85% of NPCs and 100% of gastric carcinomas (Decaussin *et al.*, 2000; zur Hausen *et al.*, 2000). EBV-positive gastric carcinomas and the majority of NPCs do not express LMP1. It has been suggested that in these cases BARF1 may be acting as the viral oncogene in the absence of LMP1. These data also suggest that in epithelial tumors BARF1 is a latent, rather than early, gene.

Late gene products

Glycoproteins

EBV encodes a number of glycoproteins of which eleven have been described. The glycoproteins present in the classical MA complex are gp350/220 (BLLF1) and gH (gp85, BXLF2) (Edson and Thorley-Lawson, 1981). The BLLF1 ORF is transcribed as two mRNA species, the smaller of which is generated by an in-frame splice such that gp220 is effectively an internally deleted version of gp340 (Biggin *et al.*, 1984). These products are highly glycosylated; around 50% of their mass is carbohydrate. The region that is spliced out of gp350 to generate gp220 contains a repetitive region consisting of a basic 21-amino-acid unit composed of three 7-amino-acid subunits. In some units, the third subunit is missing. In common with other repetitive regions of the EBV genome, the length varies between strains and has been observed to range from 56 to 126 a.a. (Lees *et al.*, 1993).

The N-terminal region of gp350/220 mediates binding of EBV to its B-cell receptor, CD21 (also known as CR2) (Fingeroth *et al.*, 1984; Nemerow *et al.*, 1989). This interaction induces capping of the receptor, endocytosis of the virus into the cell (Tanner *et al.*, 1987) and triggers tyrosine/PI kinase and NF-κB-dependent intracellular signaling pathways that activate the Wp promoter (Sinclair and Farrell, 1995; Sugano *et al.*, 1997). However, a BLLF1 deletant was able to infect B-cells and epithelial cells indicating that the gp350/220-CD21 interaction is not exclusively required for infection and that other viral proteins can mediate cellular attachment (Janz *et al.*, 2000). This function could be performed by the gH/gL/gp42 complex (see below).

gp350/220 is the major EBV neutralizing-antibody determinant (Thorley-Lawson and Poodry, 1982) and for this reason has been the focus of efforts to develop an anti-EBV vaccine (Arrand, 1992). Following encouraging human trials using a recombinant vaccinia-based delivery system (Gu *et al.*, 1991) a subunit gp340 vaccine (Jackman *et al.*, 1999) has progressed through phase 1 clinical trials and is now in phase 2 (http://www.medimmune.com/pipeline/EpsteinBarrVirusvaccine.asp).

The third component of MA, gH (BXLF2), forms a heterotrimeric complex with two more glycoproteins, gL (BKRF2) and gp42 (BZLF2). This complex is involved in the penetration of B-cells by EBV and uses cell-surface HLA class II molecules as coreceptor. It is proposed that primary binding of EBV to the B cell is mediated by the gp350/220–CD21 interaction. This is subsequently augmented by gp42 binding to HLA class II followed by virus–cell fusion mediated by the gH/gL complex (Hutt-Fletcher and Lake, 2001).

Infection of epithelial cells is somewhat different since they do not express surface HLA class II. In this case EBV uses a dimeric complex of gH-gL (without gp42) to bind to an unidentified receptor. EBV carries both dimeric and trimeric complexes in order to infect both types of host. The presence or absence of HLA class II in the virus-producing cell alters the ratio of these complexes such that epithelial cell-derived virus efficiently infects B cells and vice versa (Borza and Hutt-Fletcher, 2002).

A second EBV glycoprotein complex consists of gN (BLRF1) and gM (BBRF3). Co-expression of gM with gN is required for processing of the latter to its mature form (Lake *et al.*, 1998). Recombinant EBV in which the gN gene is inactivated fails to accumulate gM. In cells infected with this virus many of the capsids remain trapped in the nucleus and the majority of the virions are non-enveloped (Lake and Hutt-Fletcher, 2000).

The BALF4 ORF encodes gp110, a highly conserved homologue of the abundant envelope protein gB of other herpesviruses which is involved in virus–cell fusion. In contrast, EBV gB appeared to be virtually absent from the virion and to be localized to the nuclear membrane and endoplasmic reticulum (Gong and Kieff, 1990; Gong

et al., 1987; Papworth *et al.*, 1997). A recombinant EBV lacking gp110 was not released from the cell (Herrold *et al.*, 1996). These data led to the notion that the EBV gB is involved in the egress of virions from the lytically infected cell.

Recent data (Neuhierl *et al.*, 2002) indicate that B95–8 cells and virions are unusual and that in other virus strains gB is present both in the virion and on the plasma membrane of the host cell. Virions that contain gB have an enhanced infectivity and a wider cell tropism, consistent with a role in virus–cell fusion and viral entry similar to other herpesviral gBs. In keeping with this, a virus-free assay has shown that gB can mediate membrane fusion (Haan *et al.*, 2001).

EBV also encodes four poorly characterized membrane proteins; gp78 (BILF2), a highly glycosylated virion envelope glycoprotein of unknown function (Mackett *et al.*, 1990), gp150 (BDLF3), gp60 (BILF1) and BMRF2.

gp150 is an envelope glycoprotein that is ~75% carbohydrate and is non-essential for growth in B cells in vitro. A gp150-negative virus was fully competent for binding, infectivity, assembly and egress of both B cells and epithelial cells. Mysteriously, it had an enhanced infectivity for epithelial cells (Borza and Hutt-Fletcher, 1998).

gp60 and BMRF2 are both predicted to have multiple membrane-spanning domains and gp60 appears to be glycosylated (Hutt-Fletcher and Lake, 2001). BMRF2 is a 55 kD membrane protein (Modrow *et al.*, 1992) that can interact with integrins to facilitate the infection of basolateral surfaces of epithelial cells by EBV (Tugizov *et al.*, 2003).

Structural proteins

In addition to the virion glycoproteins described above, some members of the immunologically defined VCA complex have been characterized as products of specific ORFs. The major capsid protein is p160 (BcLF1) and three small capsid proteins, p18, p23 and p40 (BFRF3, BLRF2 and BdRF1) have been identified (Reischl *et al.*, 1996; van Grunsven *et al.*, 1993). The BVRF2 ORF overlaps BdRF1 and appears to encode a protease that is involved in the maturation of p40 (Donaghy and Jupp, 1995).

Studies on the protein composition of purified virions (Johannsen *et al.*, 2004) reveal that the tegument contains several late proteins including BNRF1, the major 143kD tegument component (Cameron *et al.*, 1987) and the large, 3149 a.a. tegument protein BPLF1 (Schmaus *et al.*, 2004). Interestingly a number of host cell proteins including β-actin, cofilin, tubulin, Hsp70 and Hsp90 were also present in the tegument. It is suggested that inclusion of these host cell proteins within the virion may indicate that they have a role as "mediators of morphogenesis" (Johannsen et al., 2004).

Immunofluorescent assays for VCA are still used in EBV diagnosis and, as with EA, the immunological utility of individual, purified VCA components as more objective targets has been examined. Immunogenic determinants have been associated with p160, p143, p38, p40, p18 and gB (Chen *et al.*, 1991; Hinderer *et al.*, 1999). As with novel ELISA assays using EA components, late proteins showed excellent performance in initial tests but the classical immunofluorescence assays remain in clinical use.

Viral interleukin-10

The BCRF1 ORF of EBV is highly conserved between strains (Stuart *et al.*, 1995) and exhibits extensive sequence and functional homology with the human cytokine interleukin-10 (hIL-10) (Moore *et al.*, 1990). It is therefore termed viral IL-10 (vIL-10). The mature protein sequences share 83% identity and most of their divergence is found in the N-terminal 20 amino acids. Functional activities, involving cells of the immune system, include deactivation of macrophages and dendritic cells, inhibition of cytokine synthesis by $CD4^+$T cells and proliferation and differentiation of activated B-cells (Moore *et al.*, 2001). However, some differences between hIL-10 and vIL-10 have been noted (Salek-Ardakani *et al.*, 2001).

In terms of the effects of vIL-10 on the life cycle of EBV, it has been shown that whilst vIL-10 is not absolutely required for B-cell transformation addition of exogenous vIL-10 significantly enhanced the efficiency of viral transformation of B-cells, increased cell viability and inhibited the production of antiviral interferon-γ (Stuart *et al.*, 1995; Swaminathan *et al.*, 1993). In addition, vIL-10 has been shown to counteract the inhibitory effect of T-cells on EBV-induced B cell transformation (Bejarano and Masucci, 1998). Since vIL-10 is a late gene product these observations suggest a model whereby lytically infected cells produce both virions and vIL-10. The latter acts on the surrounding cellular environment to facilitate propagation of EBV both by enhancing the susceptibility of B-cells to be transformed by the virus and by inhibiting the host's immunological defences against such action.

EBV persistence in vivo

Several lines of evidence support a role for the B lymphocyte as the site of EBV persistence in vivo. Indeed, therapy aimed at eliminating virus replication using long-term acyclovir treatment (which following conversion to

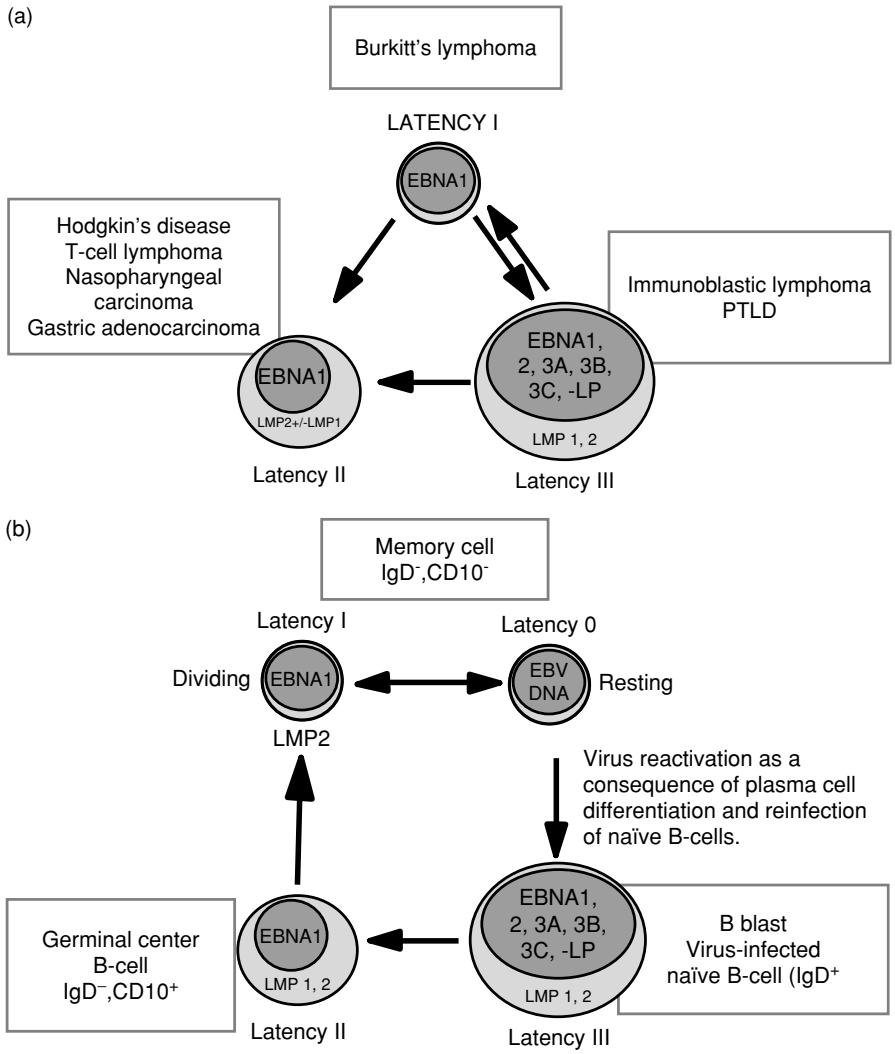

Fig. 27.6. The different forms of EBV latency manifest in virus-associated tumors (a) and during infection of normal differentiating B-cells (b). Arrows depict the interchangeable forms of latency observed by various manipulations in vitro (a) which are believed to recapitulate those occurring during B-cell differentiation in vivo (b).

acyclovir triphosphate interferes with viral replication by competing with deoxyguanosine triphosphate for viral DNA polymerase) eliminates virus excretion from the oropharnyx but does not affect the level of latent infection in B-cells (Ernberg and Andersson, 1986). As soon as treatment is halted, virus can be detected in the oropharyngeal secretions at pre-treatment levels (Yao *et al.*, 1989). In addition, studies of EBV strains in donor–recipient pairs before and after bone marrow transplantation (BMT) have shown that the recipient's strain disappeared from the oropharynx and was replaced by the donor's strain, indicating that the bone marrow B-cells harbor EBV (Gratama *et al.*, 1990). Furthermore, patients with X-linked agammaglobulinemia (XLA), who are deficient in mature B-cells, are found to be free of EBV infection, suggesting they are not able to maintain a persistent infection (Faulkner *et al.*, 1999).

EBV exists in the peripheral blood within the IgD memory B cell pool; EBV gene expression in these cells being restricted to LMP2A and possibly EBNA1 (Fig. 27.6) (Babcock *et al.*, 1998). Recent work has shown that a subset of healthy tonsils contains EBV^+ naïve (IgD^+) B-cells and that these cells express the Lat III programme and show an activated phenotype, suggesting they have been directly infected (Joseph *et al.*, 2000). These cells are

presumably either eliminated by virus-specific cytotoxic T-lymphocytes (CTLs) or differentiate to IgD memory B-cells, which then leave the tonsil. Some of these memory B-cells will pass through mucosal lymphoid tissues and terminally differentiate into plasma cells, whereupon they might enter the lytic cycle. However, a proportion also exits the cell cycle and will replenish the peripheral pool of infected memory cells. Recent work suggests that the majority of resting EBV-infected memory B-cells in vivo do not express virus latent genes and that EBNA1 expression only occurs when these cells divide (Hochberg *et al.*, 2004). The Lat II pattern of EBV gene expression has also been detected in tonsillar memory B-cells and germinal center B-cells (Babcock and Thorley-Lawson, 2000). LMP1 can provide surrogate T cell help via mimicry of an activated CD40 receptor and LMP2A can substitute for B cell receptor engagement (see later). Thus, the virus might enter a germinal center reaction and re-express LMP1 and LMP2A, providing a mechanism for the antigen-independent expansion of EBV-infected B-cells (Babcock and Thorley-Lawson, 2000; Thorley-Lawson, 2001). However, these data are not supported by studies of CD40 null mice, which are defective for isotype switching and germinal center formation. When LMP1 is constitutively expressed from a transgene in the B-cells of these mice, they are still not able to form germinal centers or produce high-affinity antibodies (Uchida *et al.*, 1999). Furthermore, germinal centers are also not formed when LMP1 is expressed in a wild-type (CD40+) background suggesting that, rather than facilitating a germinal centre reaction, LMP1 actively inhibits this process. These conflicts remain to be resolved.

Although much of the evidence described above implicates the B cell compartment as the site of persistence, a role for infection of squamous epithelial cells is suggested by the detection of EBV in oral hairy leukoplakia (Fig. 27.7), a benign lesion of the oral epithelia observed in immunosuppressed patients characterized by intense lytic infection of these tissues (De Souza *et al.*, 1989). However, EBV is not usually detectable in normal epithelial tissues, including desquamated oropharyngeal cells and tonsilar epithelium from IM patients (Karajannis *et al.*, 1997; Niedobitek *et al.*, 1989, 2000) and normal epithelium adjacent to EBV-positive UNPCs (Sam *et al.*, 1993) and gastric carcinomas (Gulley *et al.*, 1996). Therefore, it is still not clear what role, if any, epithelial cells play in the normal life cycle of the virus. It may be that epithelial infection acts to amplify EBV via virus replication during primary infection or occasionally during asymptomatic persistence and that this effect is exaggerated in hairy leukoplakia as a consequence of immunosuppression. The establishment of EBV latent infection in epithelial cells is likely to be an unusual event dependent on underlying changes within the cell that permit the stable maintenance of the virus genome (Jones *et al.*, 2003; Knox *et al.*, 1996). This latter scenario is consistent with recent studies on the genetics of NPC that demonstrate that loss of heterozygosity of certain key loci occurs as an initiating event prior to EBV infection (Lo and Huang, 2002).

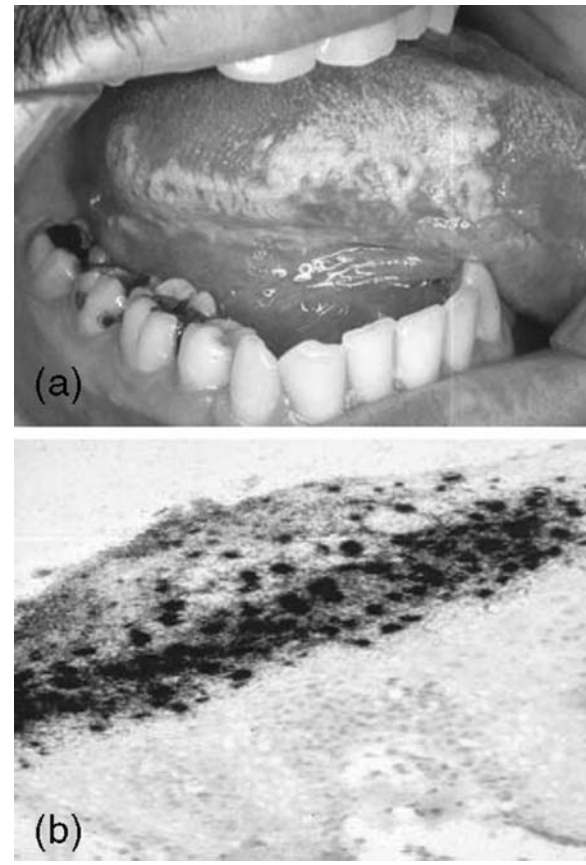

Fig. 27.7. Oral hairy leukoplakia (OHL) is a benign condition of the oral tissues that is characterized by white plaque-like lesions on the lateral tongue, as shown in (a). (b) *In situ* hybridization demonstrates the presence of abundant EBV DNA in the upper layers of the squamous epithelium of an OHL lesions consistent with virus replication.

EBV strain variation

The discovery of genetic differences between EBV carried in the Burkitt's lymphoma cell lines of West African origin, Jijoye and AG876, and the prototype B95.8 virus has led to the study of EBV strain differences. EBV isolates are characterized as type 1 (B95.8-like) or type 2 (Jijoye-and

AG876-like); these were originally referred to as A and B, respectively. EBV-1 and EBV-2 are mostly identical over the bulk of the EBV genome (Dolan *et al.*, 2006) but show allelic polymorphism (with 50–80% sequence homology depending on the locus) in a subset of latent genes, namely those encoding EBNA-LP, EBNA2, EBNA3A, EBNA3B and EBNA3C (Dambaugh *et al.*, 1984; Sample *et al.*, 1990). A combination of virus isolation and sero-epidemiological studies suggest that type 1 virus isolates are predominant (but not exclusively so) in many Western countries, whereas both types are widespread in equatorial Africa, New Guinea and perhaps certain other regions (Young *et al.*, 1987). In vitro studies show that type 1 isolates are more potent than type 2 in achieving B-cell transformation in vitro; the type 2 virus-transformed LCLs characteristically show much slower growth especially in early passage (Rickinson and Kieff, 2001). In addition to this broad distinction between EBV types 1 and 2, there is also minor heterogeneity within each virus type. Individual strains have been identified on the basis of changes, compared with B95.8, ranging from single base mutations to extensive deletions. Of particular note is the 11.8 kb deletion in the BamHIA region of the B95.8 strain (Fig. 27.1) which further emphasizes that this strain is not generally representative of the majority of naturally occurring isolates.

It was originally believed that in contrast to most immunologically compromised patients, who were shown to be infected with multiple EBV strains (Yao *et al.*, 1996b, 1998), the majority of healthy individuals are only infected with one virus type (Yao *et al.*, 1991). This led to the suggestion that the immune response to an existing EBV strain may protect the immunocompetent host from infection with additional exogenous viral strains. However, there is increasing evidence that immunocompetent carriers may also be infected with two or more separate EBV strains. For example, in one recent study that utilized the host cytotoxic T-lymphocyte response to a polymorphic EBV epitope as an indicator of the resident virus strains, 3/15 EBV-seropositive donors harbored more than one virus strain (Brooks *et al.*, 2000). A further study, which examined EBV strain variation in collections of peripheral blood mononuclear cells and multiple lymphoid and epithelial tissues of EBV-positive carriers, also demonstrated that immunocompetent individuals frequently harbor at least two strains (Srivastava *et al.*, 2000). More recent work using novel genotyping techniques confirmed that many healthy individuals harbor multiple EBV isolates and that their relative abundance and presence appears to vary over time (Sitki-Green *et al.*, 2003; Walling *et al.*, 2003).

An interpretation of the more recent data is that both immunocompromised persons and immunocompetent normal carriers are infected with multiple EBV strains, but that the rarer strains are more readily detectable in immunosuppressed individuals probably because of a higher viral load. This also implies that the immune response to an existing infection might not protect the host from subsequent infection with additional exogenous viral strains. Coinfection of the host with multiple virus strains could have evolutionary benefit to EBV enabling the generation of diversity by genetic recombination. Such intertypic recombination has been demonstrated in HIV-infected patients and appears to arise via recombination of multiple EBV strains during the intense EBV replication that occurs as a consequence of immunosuppression (Walling and Raab-Traub, 1994; Yao *et al.*, 1996a).

More contentious is the possible contribution of EBV strain variation to virus-associated tumors. Many studies have failed to establish an epidemiological association between EBV strains and disease and suggest that the specific EBV gene polymorphisms detected in virus-associated tumors occur with similar frequencies in EBV isolates from healthy virus carriers from the same geographic region (Edwards *et al.*, 1999; Khanim *et al.*, 1996). However, this does not exclude the possibility that variation in specific EBV genes is responsible for the distinct geographic distribution of virus-associated malignancies. In this regard, an LMP1 variant containing a 10 amino acid deletion (residues 343 to 352) was originally identified in Chinese NPC biopsies and has oncogenic and other functional properties distinct from those of the B95.8 LMP1 gene (Dawson *et al.*, 2000; Fielding *et al.*, 2001; Li *et al.*, 1996; Miller *et al.*, 1998). It is therefore likely that variation in LMP1 and other EBV genes can contribute to the risk of developing virus-associated tumors but more biological studies using well-defined EBV variants are required.

Function of the EBV latent genes: from persistence to pathology

An understanding of EBV latent gene function is relevant both to the factors contributing to the establishment of persistent infection in the memory B-cell pool and to the role of the virus in the oncogenic process. The advent of recombinant EBV technology has confirmed the absolute requirement for EBNA2 and LMP1 in the in vitro transformation of B-cells and highlighted a role for EBNA-LP, EBNA3A, EBNA3C and LMP2A in this process (Kieff and Rickinson, 2001). These studies confirm that EBV-induced B cell transformation requires the coordinate action of several latent genes but do not address the consequences of the more restricted patterns of EBV latent gene expression

observed in persistent infection and in certain EBV-associated tumors. More recent studies using recombinant EBV to infect either virus-negative BL cell lines or epithelial cell lines are beginning to define the contribution of more limited EBV latent gene expression to the cell phenotype and to dissect the mechanisms responsible for regulating virus gene expression in different cellular environments. A brief description of EBV latent gene function follows but other Chapters will provide a more comprehensive review of this area.

EBNA1

EBNA1 is a DNA-binding protein that is required for the replication and maintenance of the episomal EBV genome; a function that is achieved through the binding of EBNA1 to oriP, the plasmid origin of viral replication (Kieff and Rickinson, 2001). EBNA1 also interacts with two sites downstream of Qp to negatively regulate its own expression (Nonkwelo *et al.*, 1996). EBNA1 also acts as a transcriptional transactivator and up-regulates Cp and the LMP1 promoter (Kieff and Rickinson, 2001). The EBNA1 protein contains a glycine–glycine–alanine (gly–gly–ala) repeat sequence, which varies in size in different EBV isolates. This gly–gly–ala repeat domain is a *cis*-acting inhibitor of MHC class I-restricted presentation and appears to function by inhibiting antigen processing via the ubiquitin-proteosome pathway (Levitskaya *et al.*, 1995). Failure to present EBNA1-derived peptides results in ineffective CD8+ T-cell responses to EBNA1 when expressed in target cells. Directing EBNA1 expression to B-cells in transgenic mice results in B-cell lymphomas suggesting that EBNA1 might also have a direct role in oncogenesis (Wilson *et al.*, 1996). On the contrary, similar experiments using a different strain of mice failed to reveal any EBNA1-induced lymphomagenesis (Kang *et al.*, 2005). Furthermore, experiments using an EBNA1-negative EBV are also consistent with EBNA1 not being oncogenic: EBNA1 is not essential for the immortalisation of B cells by EBV and such immortalised cells have similar tumourigenic properties in SCID mice as do B-cells immortalised with wild-type, EBNA1-positive EBV (Humme *et al.*, 2003). However, a previous study demonstrates that EBNA1 is toxic in certain epithelial cell environments and that this is associated with the processing and presentation of EBNA1 to specific CTLs (Jones et al., 2003). This suggests that the establishment of EBV latency is dependent on the cell background and that CTL responses to EBNA1 may contribute to the control of latent infection. EBNA1 has recently been shown to interact with an ubiquitin-specific protease called USP7 or HAUSP that has been previously implicated in the stabilisation of p53and this may account for the ability of EBNA1 to protect B cells from apoptosis (Kennedy *et al.*, 2003; Saridakis *et al.*, 2005). Given these effects and the ability of EBNA1 to function as a transcriptional activator of several viral genes, it is likely that EBNA1 will also be able to influence cellular gene expression.

EBNA2

The inability of an EBV strain, P3HR-1, carrying a deletion of the gene encoding EBNA2 and the last two exons of EBNA-LP, to transform B cells *in vitro* was the first indication of the crucial role of the EBNA2 protein in the transformation process (Kieff and Rickinson, 2001). Restoration of the EBNA2 gene into P3HR-1 has unequivocally confirmed the importance of EBNA2 in B-cell transformation and has allowed the functionally relevant domains of the EBNA2 protein to be identified (Hammerschmidt and Sugden, 1989; Rabson *et al.*, 1982). EBNA2 is a transcriptional activator of both cellular and viral genes, and up-regulates the expression of certain B-cell antigens, including CD21 and CD23, as well as LMP1 and LMP2 (Kieff and Rickinson, 2001; Wang *et al.*, 1990). EBNA2 also transactivates the Cp promoter thereby inducing the switch from Wp to Cp observed early in B-cell infection. EBNA2 interacts with a ubiquitous DNA-binding protein, RBP-Jκ, and this is partly responsible for targeting EBNA2 to promoters that contain the RBP-Jκ sequence (Grossman *et al.*, 1994). The RBP-Jκ homologue in *Drosophila* is involved in signal transduction from the Notch receptor, a pathway that is important in cell fate determination in *Drosophila* and has also been implicated in the development of T-cell tumors in humans (Artavanis-Tsakonas *et al.*, 1995). Recent work demonstrates that EBNA2 can functionally replace the intracellular region of Notch (Hofelmayr *et al.*, 2001; Sakai *et al.*, 1998; Strobl *et al.*, 2000). The c-*myc* oncogene is also a transcriptional target of EBNA2; an effect that is likely to be important for EBV-induced B-cell proliferation (Kaiser *et al.*, 1999).

EBNA3 family

Studies with EBV recombinants have demonstrated that EBNA3A and EBNA3C are essential for B-cell transformation in vitro, whereas EBNA3B is dispensable (Robertson, 1997). EBNA3C can induce the up-regulation of both cellular (CD21) and viral (LMP1) gene expression (Allday and Farrell, 1994), repress the Cp promoter (Radkov *et al.*,

1997), and might interact with the retinoblastoma protein, pRb, to promote transformation (Parker *et al.*, 1996). While not essential for transformation, EBNA3B has been shown to induce expression of vimentin and CD40 (Silins and Sculley, 1994). The EBNA3 proteins associate with the RBP-Jκ transcription factor and disrupt its binding to the cognate Jκ sequence and to EBNA2, thus repressing EBNA2-mediated transactivation (Robertson, 1997). Thus, EBNA2 and the EBNA3 proteins work together to precisely control RBP-Jκ activity, thereby regulating the expression of cellular and viral promoters containing Jκ cognate sequence. EBNA3C has been shown to interact with human histone deacetylase 1, which, in turn, contributes to the transcriptional repression of Cp, by RBP-Jκ (Radkov *et al.*, 1999).

EBNA-LP

EBNA-LP is encoded by the leader of each of the EBNA mRNAs and encodes a protein of variable size depending on the number of *Bam*HI W repeats contained by a particular EBV isolate. The precise role of EBNA-LP in B-cell transformation *in* vitro is not clear but EBNA-LP is required for the efficient outgrowth of LCLs (Allan *et al.*, 1992). Transient transfection of EBNA-LP and EBNA2 into primary B-cells induces G0 to G1 transition as measured by the up-regulation of cyclin D2 expression (Sinclair *et al.*, 1994). EBNA-LP can also cooperate with EBNA2 in up-regulating transcriptional targets of EBNA2, including LMP1 (Harada and Kieff, 1997; Nitsche *et al.*, 1997). EBNA-LP has been shown to colocalize with pRb in LCLs and in vitro biochemical studies have demonstrated an interaction of EBNA-LP with both pRb and p53 (Jiang *et al.*, 1991; Szekely *et al.*, 1993). However, this interaction has not been verified in LCLs and, unlike the situation with the human papillomavirus (HPV)-encoded E6/E7 and adenovirus E1 proteins, EBNA-LP expression appears to have no effect on the regulation of the pRb and p53 pathways.

LMP1

LMP1 is the major transforming protein of EBV behaving as a classical oncogene in rodent fibroblast transformation assays and being essential for EBV-induced B-cell transformation in vitro (Kieff and Rickinson, 2001). LMP1 has pleiotropic effects when expressed in cells resulting in induction of cell surface adhesion molecules and activation antigens (Wang *et al.*, 1990), up-regulation of anti-apoptotic proteins (Bcl-2, A20) (Henderson *et al.*, 1991; Laherty *et al.*, 1992) and stimulation of cytokine production (IL-6, IL-8) (Eliopoulos *et al.*, 1997, 1999). Recent studies have demonstrated that LMP1 functions as a constitutively activated member of the tumor necrosis factor receptor (TNFR) superfamily activating a number of signaling pathways in a ligand-independent manner (Gires *et al.*, 1997; Kilger *et al.*, 1998). Functionally, LMP1 resembles CD40, a member of the TNFR, and can partially substitute for CD40 in vivo providing both growth and differentiation responses in B-cells (Uchida *et al.*, 1999). The LMP1 protein is an integral membrane protein of 63 kD and can be subdivided into three domains: (a) a N-terminal cytoplasmic tail (amino acids 1–23) which tethers and orientates the LMP1 protein to the plasma membrane, (b) six hydrophobic transmembrane loops which are involved in self aggregation and oligomerization (amino acids 24–186); (c) a long C-terminal cytoplasmic region (amino acids 187–386) which possesses most of the molecule's signaling activity. Two distinct functional domains referred to as C-terminal activation regions 1 and 2 (CTAR1 and CTAR2) have been identified on the basis of their ability to activate the NF-κB transcription factor pathway (Huen *et al.*, 1995). This effect contributes to the many phenotypic consequences of LMP1 expression including the induction of various anti-apoptotic and cytokine genes. More recent work demonstrates that LMP1 can also regulate the processing of p100 NF-κB2 to p52 and that this is independent of the pathways responsible for controlling the canonical NF-κB pathway (Eliopoulos *et al.*, 2003). LMP1 is also able to engage the MAP kinase cascade resulting in activation of ERK, JNK and p38 and to stimulate the JAK/STAT pathway (Eliopoulos *et al.*, 1999; Eliopoulos and Young, 1998; Gires *et al.*, 1999; Kieser *et al.*, 1997; Roberts and Cooper, 1998). Many of these effects result from the ability of TNFR-associated factors (TRAFs) to interact either directly with CTAR1 or indirectly via the death domain protein TRADD to CTAR2. The binding of TRAFs to the multimerized cytoplasmic tails of LMP1 provides a platform for the assembly and activation of upstream signaling molecules including the NIK and Tpl-2 MAPK kinase kinases (Eliopoulos *et al.*, 2002; Sylla *et al.*, 1998). The precise mechanisms responsible for signal initiation from these multiprotein complexes remain unknown. The region between CTAR1 and CTAR2 (so-called CTAR3) has been suggested to be responsible for the JAK/STAT pathway, although other data refute this finding and deletion of this region has no effect on the efficiency of B-cell transformation (Gires *et al.*, 1999; Higuchi *et al.*, 2002; Izumi *et al.*, 1999). Recent work has also demonstrated that LMP1 can also activate the phosphatidylinositol 3-kinase (PI3-K) pathway resulting in a variety of effects including cell survival mediated through the Akt (PKB) kinase,

actin polymerisation and cell motility (Dawson *et al.*, 2003).

LMP2

The gene encoding LMP2 yields two distinct proteins, LMP2A and LMP2B. The structures of LMP2A and LMP2B are similar; both have 12 transmembrane domains and a 27 amino acid cytoplasmic C-terminus (Kieff and Rickinson, 2001). In addition, LMP2A has a 119 amino acid cytoplasmic amino-terminal domain. LMP2A aggregates in patches within the plasma membrane of latently infected B-cells (Longnecker and Kieff, 1990). Neither LMP2A nor LMP2B are essential for B-cell transformation (Longnecker, 2000). The LMP2A amino-terminal domain contains eight tyrosine residues, two of which (Y74 and Y85) form an immunoreceptor tyrosine-based activation motif (ITAM) (Fruehling and Longnecker, 1997). When phosphorylated, the ITAM present in the B-cell receptor (BCR) plays a central role in mediating lymphocyte proliferation and differentiation by the recruitment and activation of the *src* family of protein tyrosine kinases (PTKs) and the *syk* PTK. LMP2A also interacts with these PTKs through its phosphorylated ITAM and this association appears to negatively regulate PTK activity (Fruehling and Longnecker, 1997). Thus, the LMP2A ITAM has been shown to be responsible for blocking BCR-stimulated calcium mobilization, tyrosine phosphorylation and activation of the EBV lytic cycle in B cells (Miller *et al.*, 1995).

Expression of LMP2A in the B cells of transgenic mice abrogates normal B cell development allowing immunoglobulin-negative cells to colonize peripheral lymphoid organs (Caldwell *et al.*, 1998). This suggests that LMP2A can drive the proliferation and survival of B-cells in the absence of signaling through the BCR. Taken together, these data support a role for LMP2 in modifying the normal programme of B-cell development to favor the maintenance of EBV latency and to prevent inappropriate activation of the EBV lytic cycle. A modulatory role for LMP2B in regulating LMP2A function has been suggested (Longnecker, 2000). The consistent expression of LMP2A in HD and NPC suggests an important function for this protein in oncogenesis but this remains to be shown. LMP2A also recruits Nedd4-like ubiquitin protein ligases; this might promote *lyn* and *syk* ubiquitination in a fashion that contributes to a block in B-cell signaling (Ikeda *et al.*, 2000). Furthermore, a recent report shows that LMP2A can transform epithelial cells and that this effect is mediated, at least in part, by activation of the PI3-kinase/Akt pathway (Scholle *et al.*, 2000). This suggests that LMP2A-induced activation of the Akt pathway may be relevant to the long-term survival of persistently infected memory B-cells.

EBERs

In addition to the latent proteins, two small non-polyadenylated (non-coding) RNAs, EBERs 1 and 2 are probably expressed in all forms of latency. However, the EBERs are not essential for EBV-induced transformation of primary B-lymphocytes (Swaminathan *et al.*, 1991).

The EBERs assemble into stable ribonucleoprotein particles with the auto-antigen La (Lerner *et al.*, 1981), with ribosomal protein L22 (Toczyski *et al.*, 1994) and bind the interferon-inducible, double-stranded RNA-activated protein kinase PKR (Clemens *et al.*, 1994). PKR has a role in mediating the antiviral effects of the interferons and it has been suggested that EBER-mediated inhibition of PKR function could be important for viral persistence perhaps by protecting cells from interferon induced apoptosis (Nanbo *et al.*, 2002). Studies in transfected NIH 3T3 cells demonstrated that EBER1 can enhance protein synthesis by a PKR-independent mechanism (Laing *et al.*, 2002).

Reintroduction of the EBERs into EBV-negative Akata BL cells restores their capacity for growth in soft agar, tumorigenicity in SCID mice and resistance to apoptotic inducers; features identical to those observed in the parental EBV-positive Akata cells (Komano *et al.*, 1999). The detection of IL-10 expression in EBV-positive, but not in EBV negative, BL tumors and the observation that the EBERs can induce IL-10 expression in BL cell lines, suggests that IL-10 may be an important component in the pathogenesis of EBV-positive BL (Kitagawa *et al.*, 2000). Recently, it has been shown that stable expression of bcl-2 or the EBERs in EBV-negative Akata cells significantly enhanced the tumourigenic potential of these cells, but neither bcl-2 nor the EBERs restored tumorigenicity to the same extent as EBV (Ruf *et al.*, 2000). Overall, these studies suggest that EBV genes previously shown to be dispensable for transformation in B-cell systems (e.g., EBERs) might make more important contributions to the pathogenesis of some EBV-associated malignancies and to EBV persistence than was previously appreciated.

BARTs

The BARTs were first identified in NPC tissue (Hitt *et al.*, 1989) and subsequently in other EBV-associated malignancies such as BL (Tao *et al.*, 1998), HD (Deacon *et al.*, 1993) and nasal T-cell lymphoma (Chiang *et al.*, 1996) as well as

in the peripheral blood of healthy individuals (Chen *et al.*, 1999). The BARTs encode a number of potential open reading frames (ORFs) including BARF0, RK-BARF0, A73 and RPMS1. The protein products of these ORFs have not be identified and remain controversial (van Beek *et al.*, 2003). However, in vitro studies have suggested potential functions including the negative regulation of EBNA2 and Notch activity (RPMS1) and the modulation of kinase signaling (A73) (Smith *et al.*, 2000).

MicroRNAs

Micro RNAs (miRs) are small, noncoding RNA molecules of only 21–24 nucleotides in length and have been shown to play a role in the posttranscriptional downregulation of target mRNAs (Bartel, 2004; Du and Zamore, 2005; Kim and Nam, 2006). Eukaryotic cells express large numbers of miRs, often in a cell- or tissue-specific manner; indeed the July 2006 release of the microRNA database, miRBase, (Griffiths-Jones *et al.*, 2006; http://microrna.sanger.ac.uk) lists 462 human miRs. Cellular miRs are transcribed by RNA polymerase II into a long precursor molecule (primary miRNA) containing one or a cluster of several miRs. This primary transcript is then processed and transported to the cytoplasm by a series of enzymatic mechanisms (Bartel, 2004; Du and Zamore, 2005). The resulting active complex containing the mature miR is called an RNA-induced silencing complex (RISC) (Bartel, 2004; Du and Zamore, 2005; Kim and Nam, 2006). If the RISC encounters an mRNA containing extensive homology to the miR within the complex then the mRNA is degraded. Alternatively, and probably more commonly, the RISC will bind to an mRNA with only partial complementarity in which case translational repression ensues (Bartel, 2004; Du and Zamore, 2005).

Herpesviruses also enode miRs: HSV1, MHV68, hCMV, KSHV, rLCV and EBV each encode at least 2, 9, 11, 12, 16 and 23 species respectively (Cullen, 2006; Gupta *et al.*, 2006; Cui *et al.*, 2006). The EBV miRs are arranged in two clusters within the viral genome: three adjacent to the BHRF1 gene (the BHRF1 cluster) and the BART cluster which comprises the remaining 20 miRs located in the introns of the BART transcripts. (Pfeffer *et al.*, 2004; Cai *et al.*, 2006; Grundhoff *et al.*, 2006).

Intriguingly, the two clusters of EBV-encoded miRs are differentially expressed in cells exhibiting different forms of EBV latency. The BART cluster is expressed predominantly in latency I or II whereas the BHRF1 miRs are associated with latency III and Cp/Wp promoter usage. This suggests that the BHRF1 miRs may be processed from an intron in the large EBNA primary transcripts (Cai *et al.*, 2006; Cullen, 2006). The expression levels of several miRs from both clusters are enhanced following induction of lytic infection (Cai *et al.*, 2006). The significance of all these differential expression profiles is currently unclear.

The function of EBV-encoded miRs is also unclear. miR-BART2 is antisense to the BALF5 open reading frame and has been proposed to have a role in the regulation of viral DNA polymerase expression via the degradative mechanism (Pfeffer *et al.*, 2004). The rest of the miRs do not exhibit such extensive homology with EBV genes leading to the hypothesis that they are more likely to target cellular genes by translational inhibition.

Conclusions

Compelling evidence implicates EBV in the pathogenesis of tumors arising in both lymphoid and epithelial tissues. The virus adopts different forms of latent infection in different tumor types recapitulating the forms of EBV latency used to establish long-term persistence in the memory B-cell pool. These forms of latency reflect the complex interplay between EBV and the host cell environment involving differential regulation by both viral and cellular factors of the promoters driving latent gene transcription. Superimposed on this intrinsic regulation of EBV latency are extrinsic factors such as the immune response and the local milieu that may influence the growth and survival of EBV-infected cells as well as govern their entry into the virus lytic cycle. Studies of the function of individual EBV latent and lytic genes have highlighted the ability of these proteins to target specific cellular pathways and more detailed analysis using recombinant forms of EBV offers the possibility of precisely defining the role of these viral genes in the EBV life cycle. Thus, as clearly evidenced by work with proteins encoded by other viruses, an understanding of the functions of EBV proteins will not only be relevant to the role of the virus in health and disease but will also help to elucidate the fundamental mechanisms regulating cell growth, survival and differentiation. It is hoped that this work will also provide novel approaches to the therapy of EBV-associated diseases. Adoptive transfer of EBV-specific CTLs has already proved useful in the treatment of immunoblastic B-cell lymphomas and this approach as well as other vaccine strategies are currently being evaluated in patients with HD or NPC. The possibility of more direct therapeutic intervention targeting the function of essential EBV latent genes such as EBNA1 and LMP1 is also a possibility. Thus, drugs that prevent the ability of EBNA1 to bind to oriP or of the TRAFs to interact with LMP1 are likely to be developed. Finally,

gene therapy strategies which exploit either the transcriptional regulation of the EBV genome or target the functional effects of EBV latent genes offer exciting possibilities for the development of both therapeutic and preventative strategies.

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28

KSHV gene expression and regulation

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Introduction

In this chapter, both in vivo and in vitro KSHV viral gene expression patterns are described. Observations in both systems have been critical for the identification of viral proteins contributing to the pathogenic properties of this virus and for our appreciation of how this virus persists and replicates in the course of naturally occurring infections, the vast majority of which are asymptomatic (see *Epidemiology*). In contrast to other human herpesviruses, cell-free infection with KSHV in vitro is still inefficient and only a few studies have investigated viral gene expression following *de novo* infection. However, informative studies using *in situ* hybridization (ISH), immunohistochemistry (IHC), and various methods of transcript analysis have been carried out with stably infected, primary effusion lymphoma (PEL)-derived cell lines and, to a lesser extent, biopsy samples. Gradually, a picture on viral gene expression patterns and their regulation in different cell types is beginning to emerge.

Viral gene expression patterns in culture

PEL derived cell lines

PEL cell lines remain the most tractable system for examining KSHV viral gene expression. The vast majority of cells are infected latently and express a restricted repertoire of genes, while a small percentage (this varies from cell line to cell line, usually in the order of 1%–5%) of cells spontaneously switch into the lytic replication cycle. Lytic reactivation can be enhanced (up to 20% in some cell lines) in this system by chemical treatment with butyrate or phorbol esters. Despite the convenience of working with PEL cell lines, limitations exist: (1) if a bulk analysis of all cells in such a culture is carried out using Northern blots or RT-PCR, both the group of genuinely latent genes and that of strongly expressed, albeit only in a few cells, lytic genes may be detected, (2) chemical manipulation may have extraneous and secondary effects on viral gene expression, and (3) expression patterns in PEL B cell lines may not extend to expression patterns in KSHV infected tissues, in particular, spindle cells. With these caveats in mind, Fig. 28.1 shows an overview of the KSHV viral genome and the position of individual viral genes. The color coding reflects their expression during the different stages of lytic replication or latency. The coding is the result of a comparison of three publications that used gene arrays covering the entire viral genome (Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001; Nakamura *et al.*, 2003), and two earlier publications using Northern blots to investigate the basal expression and induction of KSHV genes in PEL cell lines following treatment with butyrate or phorbol esters (Sarid *et al.*, 1998; Sun *et al.*, 1999).

The first report to systematically analyze the expression pattern, by Northern blot, of the vast majority of KSHV genes in PEL cells before and after induction of the lytic cycle (Sarid *et al.*, 1998) distinguished three categories of viral genes: class I genes, expressed during latency and not upregulated by chemical induction; class II genes, characterized by a baseline expression of variable abundance (high, moderate, and low) before, and increased expression after, chemical induction; class III genes that are only expressed after chemical induction. Class I genes comprised those marked in red in Fig. 28.1, i.e. ORF73 (encoding LANA1), ORF72 (vCYC), and ORF71/K13 (vFLIP). Transcripts most likely related to ORFs K2 (vIL6), ORF70, K4 (vMIP-II), K5 (MIR1), K6 (vMIP-I), K7 (PAN/nut-1/T1.1 RNA), K8 (bZIP), ORF57, the vIRFs locus, and T0.7/Kaposin were classified as class II genes, as were at least another five transcripts that could not be unambiguously assigned to

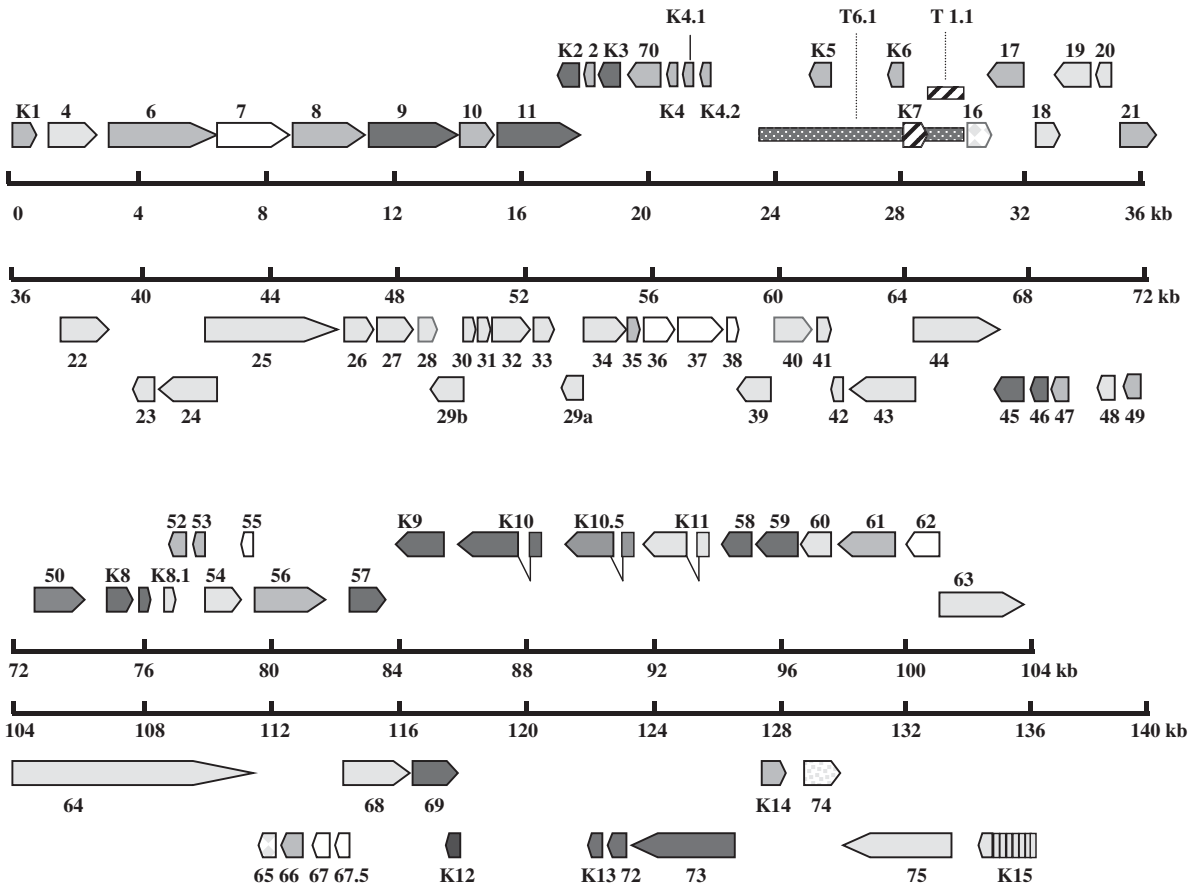

Fig. 28.1. KSHV gene expression in PEL cell lines and biopsy samples. This Figure summarizes the expression of individual KSHV genes in PEL cells during latency and following reactivation of the lytic cycle by treatment with TPA, Na-butyrate or heterologous expression of RTA by transfection or transduction. Also included are results from in situ hybridization or immunohistochemistry studies on biopsy samples of KS, MCD or PEL tumors. As discussed in the text, the color-coding is based on a comparison of several reports that studied KSHV genes by Northern blot, real time PCR or DNA array. (See color plate section.)

- Latent gene
- Latent gene in B cells only
- Latent gene in KS spindle cells in vivo; early (in some studies delayed) expression kinetics in PEL cells in vitro
- Immediate–early gene as judged by cycloheximide resistance
- Very rapid onset of gene expression in at least 2 studies
- Early lytic transcript: TPA inducible, unaffected by PFA
- Delayed onset of gene expression
- Late gene expression profile
- Late gene expression profile confirmed by PAA sensitivity
- Discrepant results in different gene array studies

individual ORFs. The remainder were considered class III genes. The existence of Class II transcripts outside of the classical dichotomous lytic-latent classification may, in part, be explain by a recent publication from H. Chang and colleagues which showed that a subset of immunoregulatory and growth promoting KSHV genes can be activated by RTA-independent notch signaling. Further, detection of these transcripts was shown to be dissociated from the full repertoire of lytic viral gene expression (Chang *et al.* 2005).

A subsequent study (Sun *et al.*, 1999) attempted to relate the pattern of viral gene expression seen following the induction of the lytic cycle by treatment with Na-butyrate or phorbol-12-myristate-13-acetate (TPA) to the conventional cascade of herpesviral gene expression

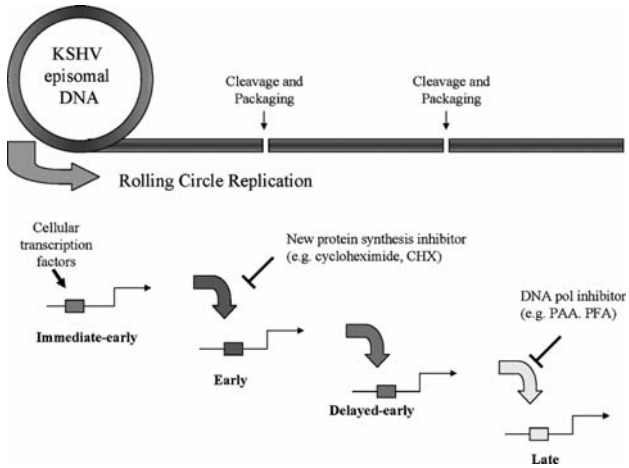

Fig. 28.2. KSHV lytic replication. Herpesvirus lytic replication is characterized by wide-spread gene activation, classically in an ordered cascade of sequential induction. The full expression of this cascade can be chemically manipulated to temporally distinguish immediate early, early and late lytic genes. Lytic activation is believed to result in a rapid increase in virion DNA as well as host cell death.

during lytic replication (Fig. 28.2). This conventional classification distinguishes immediate–early, early, and late genes: Immediate–early genes are expressed immediately following viral entry, do not require the *de novo* expression of viral proteins and are therefore resistant to treatment of the infected cell with cycloheximide. Early genes are sensitive to cycloheximide (i.e., require *de novo* protein synthesis in the infected cell) but are expressed prior to the replication of the viral genome and therefore are resistant to PAA or PFA.

Sun *et al.* (1999) classified ORF50 (RTA) as an immediate–early gene on the basis of its early onset of transcription (8 hours) following treatment with Na-butyrate and (only partial) resistance to cycloheximide. In spite of a similar rapid onset of expression seen with several other genes (e.g., h vIL6, vMIP-II, PAN/nut-1, vTS), none of these other genes examined by Sun *et al.* were resistant to cycloheximide and these therefore represent early genes. Since none of the gene array studies (see below) examined cycloheximide resistance, ORF50/RTA is therefore the only gene for which at least a partial resistance to cycloheximide has been demonstrated. It could be argued that the criterion of cycloheximide resistance should not be overinterpreted since reactivation rather than de novo infection (as in the classical experiments with alpha herpesviruses) was examined in all these studies, and that the designation of "immediate–early" for a KSHV gene on the basis of cycloheximide resistance is therefore problematic. However, ORF50/RTA stands out as the only KSHV gene with rapid expression kinetics for which this property has been demonstrated and is also the first viral gene to be expressed following *de novo* infection. Functional studies (see below and elsewhere in this book), as well as a systematic survey of viral genes whose expression is induced, directly or indirectly, by RTA (Nakamura *et al.*, 2003), have shown that RTA is the central regulator of the lytic replication cycle. It has therefore been labeled pink in Fig. 28.1.

In addition to ORF50/RTA, a few other viral genes have been shown to be resistant to cycloheximide in chemically induced PEL cell lines and thus to have immediate–early characteristics (Zhu *et al.*, 1999; Haque *et al.*, 2000; Rimessi *et al.*, 2001). In their study, Zhu *et al.* (1999) found the transcripts for ORFK8, ORF45 and ORFK4.2, in addition to that for ORF50/RTA, to have immediate-early characteristics. Haque *et al.* (2000) reported this for the ORFK5 transcript and Rimessi *et al.* (2001) for the ORFK3 mRNA.

Four groups (Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001; Nakamura *et al.*, 2003; Fakhari & Dittmer, 2002; Dittmer, 2003) have recently examined KSHV gene expression patterns in PEL cells by DNA array or real time PCR methods. All studies attempted to group individual viral genes into categories reflecting their onset or rate of expression. The results correlate on the whole, except for a few genes classified differently by several studies. These are indicated by open boxes in Fig. 28.1. All studies group ORFs 73 (LANA1), 72 (vCYC), 71/K13 (vFLIP) together as expressed in PEL cell lines prior to, and increasing only moderately following, induction of the lytic cycle ("latent" or "constitutive" genes). Based on *in situ* hybridization or immunohistochemistry, these genes, or their proteins, are expressed in the majority of infected cells in vivo (see below). These are therefore, marked in red in Fig. 28.1. As outlined below, these three viral genes are expressed from two alternatively spliced mRNAs (Fig. 28.4), with vCYC and vFLIP expressed from the same bicistronic mRNA. In spite of the general consensus that these are latent transcripts, the study by Nakamura *et al.* observed an increased expression of these mRNAs early (ORF71/ORF72) or late (ORF73) after triggering the lytic cycle by RTA expression. Since this has not been seen when Na-butyrate or phorbol esters were used to induce the lytic cycle, it may reflect the more "physiological" induction of the lytic cycle by RTA in this experiment and indicate that this group of mRNAs, although expressed during latent persistence, may increase during lytic replication.

The non-coding nuclear RNA variously referred to as PAN, nut-1 or T1.1, and T0.7 or Kaposin, one of several transcripts derived from ORFK12, were the first abundant transcripts to be identified in KS biopsies (Zhong

et al., 1996). On Northern blots from uninduced vs. induced PEL cell lines both mRNAs show basal expression that is increased upon induction of the lytic cycle (Sarid *et al.*, 1998; Sun *et al.*, 1999) and were designated as class II transcripts by Sarid *et al.* In a gene array study, Jenner *et al.* found that the expression kinetics of T0.7/Kaposin resembled that of ORFK10 (these were designated latent/lytic transcripts), while that of T1.1/PAN clustered with those of ORFK7, ORFK14 and other early genes, grouped together as "primary lytic" genes. However, in a similarly designed study by Paulose-Murphy *et al.* (2001) and in that by Nakamura *et al.* (2003) T0.7/Kaposin and K10 did not group together, with T0.7/Kaposin having later expression kinetics. Similarly, T1.1/PAN, ORFK7, ORFK14 did not group together in these two studies. We therefore chose a separate color coding in Fig. 28.1 for T0.7/ Kaposin to reflect its probable latent nature in KS spindle cells in vivo. In spite of the controversial expression kinetics for T 1.1/PAN and K7, reflected in a hatched box in Fig. 28.1, it is clear that both are lytic genes.

Recently, in the region encompassing both T1.1/PAN and K7, Taylor *et al.* (2005) found a large transcript of approximately 6.1 kb by northern blot hybridization designated T6.1. This transcript is inducible with TPA, but is resistant to PFA. RACE identified the 5′ end of the transcript at nucleotide 23,586, and 5 excised clones from a screening of a PEL cDNA lambda phage library identified the 3′ end of the transcript at nucleotide 29,741 making the transcript co-terminal with T1.1/PAN (Fig. 28.1). This transcript encompasses K7 in the same orientation and ORFs K5 and K6 in the reverse orientation. Thus far, this 6.1 kb transcript is the largest found in the KSHV genome, but the presence of large lytic transcripts is not unique to KSHV and have been reported for other herpesviruses. Smuda *et al.* (1997) found high molecular mass overlapping early lytic transcripts of 6 kb, 8 kb, 10 kb, and 14 kb within the human cytomegalovirus genome. Wirth *et al.* (1989) identified a 6 kb immediate–early transcript and six late lytic transcripts ranging from 4.5 kb to >8 kb in bovine herpesvirus 1. It is unclear why these herpesviruses produce such large transcripts, although it has been postulated that their size may lead to RNA stability by the formation of pseudoknots. Regardless of function, the presence of this large transcript has implications in the examination of expression patterns for overlapping ORFs if DNA array and real time PCR methods are used.

The KSHV genome contains a region encoding a series of proteins with homologies to cellular interferon regulatory factors (IRFs). In this region one spliced gene, K10.5, shows latent gene expression in PEL cell lines in vitro (Rivas *et al.*, 2001; Cunningham *et al.*, 2003) and the corresponding protein, LANA2 or vIRF3, has been demonstrated by immunohistochemistry in all infected B cells in MCD and PEL in vivo, suggesting a B-lineage specific latent expression pattern (Rivas *et al.*, 2001). However, the study by Nakamura *et al.* (2003) indicates that its expression is increased at a late stage following activation of the lytic cycle by RTA in a PEL cell line. Because of its B-cell specific expression pattern, K10.5/vIRF3 has been given its own (light brown) color coding in Fig. 28.1.

Three other genes in this locus, ORFs K9, K10, K11, are induced after activation of the lytic cycle (Cunningham *et al.*, 2003; Nakamura *et al.*, 2001; Paulose-Murphy *et al.*, 2001; Jenner *et al.*, 2001). Jenner *et al.* (2001) classified ORFK10, encoding vIRF4, as a "latent/lytic" gene, since its expression kinetics was similar to that of the T0.7/Kaposin transcript. The two other published gene array studies (Paulose-Murphy *et al.*, 2001; Nakamura *et al.*, 2003) concur to the extent that the ORFK10 transcript increases early after induction of the lytic cycle but group it with several other early transcripts. We have based our color coding of this gene on their results. In a similar manner, ORFK9 appears to be expressed relatively early, while in comparison ORFK11 expression appears to come on somewhat later. The upstream exon of K11, termed K11.1 in the study by Nakamura *et al.* (2003) and referred to as vIRF2 by Jenner *et al.* (2001), was classified in an earlier expression group than the second K11 exon by Nakamura *et al.* (2003), but in the same group as the second exon by Jenner *et al.* (2001).

Although the three gene array studies published so far do not always concur on the expression kinetics of individual viral genes, as illustrated by the above examples, there is broad agreement that viral proteins required for DNA replication or gene expression are produced earlier in the lytic cycle than viral structural proteins necessary for assembly of new virions. Likewise, lytic viral proteins known to be expressed in a slightly higher number of productively infected cells *in vivo* such as vIL6 or PF8 (see below) appear to be encoded by transcripts expressed in PEL cells early during the lytic cycle, whereas a structural glycoprotein encoded by ORFK8.1 and expressed only in few cells in vivo has been grouped with late transcripts. However, it needs to be emphasized that other factors than the stage of the lytic cycle may affect the expression of certain genes, e.g., vIL6, in vivo. However, in the case of vGPCR, the viral homologue of a G-coupled receptor that has been proposed to play an important role in KS pathogenesis by virtue of its ability to induce the secretion of VEGF and other paracrine factors from infected cells, the available data are controversial. While its expression kinetics in PEL cells resembled that of an early gene in the study by Jenner *et al.* and Nakamura *et al.*, Paulose-Murphy *et al.* found a delayed onset and Sun *et al.* (1999) showed that its transcript was at least partially

sensitive to PAA, suggesting late viral gene expression. However, using Northern blots Kirshner *et al.* (1999) did not see an inhibition of the vGPCR mRNA by PAA and classified it as an early lytic gene.

Viral gene expression in newly infected cultured endothelial, epithelial or fibroblast cultures

Experiments with KSHV released from PEL cell lines after chemical induction, or with recombinant KSHV preparations induced in Vero or 293 cells, have shown that KSHV can infect a wide variety of cultured cells belonging to several lineages (endothelial, epithelial, fibroblast) with the notable exception of lymphoid cell lines (Bechtel *et al.*, 2003; Lagunoff *et al.*, 2002; Moses *et al.*, 1999; Vieira *et al.*, 1997; Renne *et al.*, 1998; Gao *et al.*, 2003; Ciufo *et al.*, 2001). It is not know at this time why B lymphocytes are resistant to de novo infection, however, Chen and Lagumoff have shown that naked DNA of a KSHV BAC, engineered with a selectable element, can be introduced into BJAB cells such that virus can be maintained in latency as well as undergo lytic reactivation (Chen and Lagunoff, 2005). In the majority of these reports, KSHV quickly established a latent infection, as defined by the expression of LANA1, in most infected cells. Only a small proportion of infected cells express viral transcripts or proteins characteristic of the lytic replication cycle such as ORF59/PFA, the K8.1 envelope glycoprotein, or the minor capsid protein mCP/SCIP encoded by ORF65 (Renne *et al.*, 1998; Lagunoff *et al.*, 2002; Ciufo *et al.*, 2001; Vieira *et al.*, 1997; Moses *et al.*, 1999; Bechtel *et al.*, 2003). In these latently KSHV-infected cells the lytic replication cycle can be reactivated using phorbol esters (as in PEL cell lines), or by superinfection with CMV (Vieira *et al.*, 1997), and expression of RTA by transfection or transduction can do so in all cell types examined (Bechtel *et al.*, 2003). A possible interpretation of these findings is that events upstream and controlling the expression of RTA are responsible for the blocked lytic replication and default latency in these cells. As discussed above, methylation of CpG residues in the ORF50/RTA promoter may represent such an event (Chen *et al.*, 2001).

In one experimental system, which used a recombinant KSHV carrying a bacterial artificial chromosome and GFP cassette between ORFs 18 and 19, spontaneous transient lytic activation of KSHV in newly infected endothelial cells was observed during the first week of culture which subsided subsequently, giving way to long-term latent infection (Gao *et al.*, 2003). Lytic reactivation in this experimental system was measured by the expression of the minor capsid protein SCIP, encoded by ORF65, and latent infection by expression of LANA1 (Gao *et al.*, 2003).

Investigating early events following virus entry Dezube *et al.* (2002) found that rapid circularization of the viral genome occurred within 8 hours of infecting cultured endothelial cells with PEL-line derived KSHV. This was followed by the appearance of linear genomes, indicating lytic replication, approximately 72 hours after infection. Expression of the lytic transcripts T1.1/PAN and K8.1 could be detected by Northern blot at 3–6 days, and increased up to days 8–10, after infection. Latent transcripts for ORF72 (vCYC) and ORF73 (LANA1) were first detected on day 8. Interestingly, ORF74 (vGPCR) mRNA expression oscillated between days 1–8 (Dezube *et al.*, 2002).

Viral gene expression early after *de novo* infection of endothelial cell and fibroblast cultures has also been investigated using a KSHV microarray (Krishnan *et al.*, 2004). Following *de novo* infection, a limited number of KSHV genes are initially expressed (Fig. 28.3, Krishnan *et al.*, 2004). However, with the exception of the latent transcripts for LANA1, vCYC and vFLIP, all mRNAs are down-regulated over the following 24 hours and KSHV therefore quickly adopts a transcriptional latency pattern. The first viral transcript to be expressed following *de novo* infection encodes ORF 50/RTA, the central regulator of the viral lytic replication cycle (Krishnan *et al.*, 2004). However, only a limited number of RTA-activated viral genes (see below) show a transient expression. These include the genes for vIL6 (ORFK2), vMIP-II (ORFK4), MIR2 (ORFK5), vMIP-I (ORFK6), vIRF2 (ORF11) and the survivin homologue (ORFK7), which are all thought to play a role in modulating the response to interferon, cytotoxic T-lymphocytes, NK cells or apoptosis. In contrast, many KSHV genes involved in DNA synthesis or encoding structural proteins were not expressed. This pattern of viral gene expression suggests that, unlike α- or β-herpesviruses, KSHV quickly adopts a latent program of gene expression and only transiently expresses a set of genes that counteract the effects of the interferon system – which is induced in endothelial and fibroblast cells very early following KSHV infection (Naranatt *et al.*, 2004) – or other components of the innate or adaptive immune system. Which viral or cellular factors determine this switch into latency and interfere with the completion of the full lytic transcription program is not understood at present and remains one of the most interesting aspects of the control of KSHV gene expression.

Viral gene expression in vivo

Despite the relative ease of working with KSHV infected PEL-derived cell lines, a major problem of studying viral gene expression in such a system is that expression patterns may not reflect that found in infected tissues.

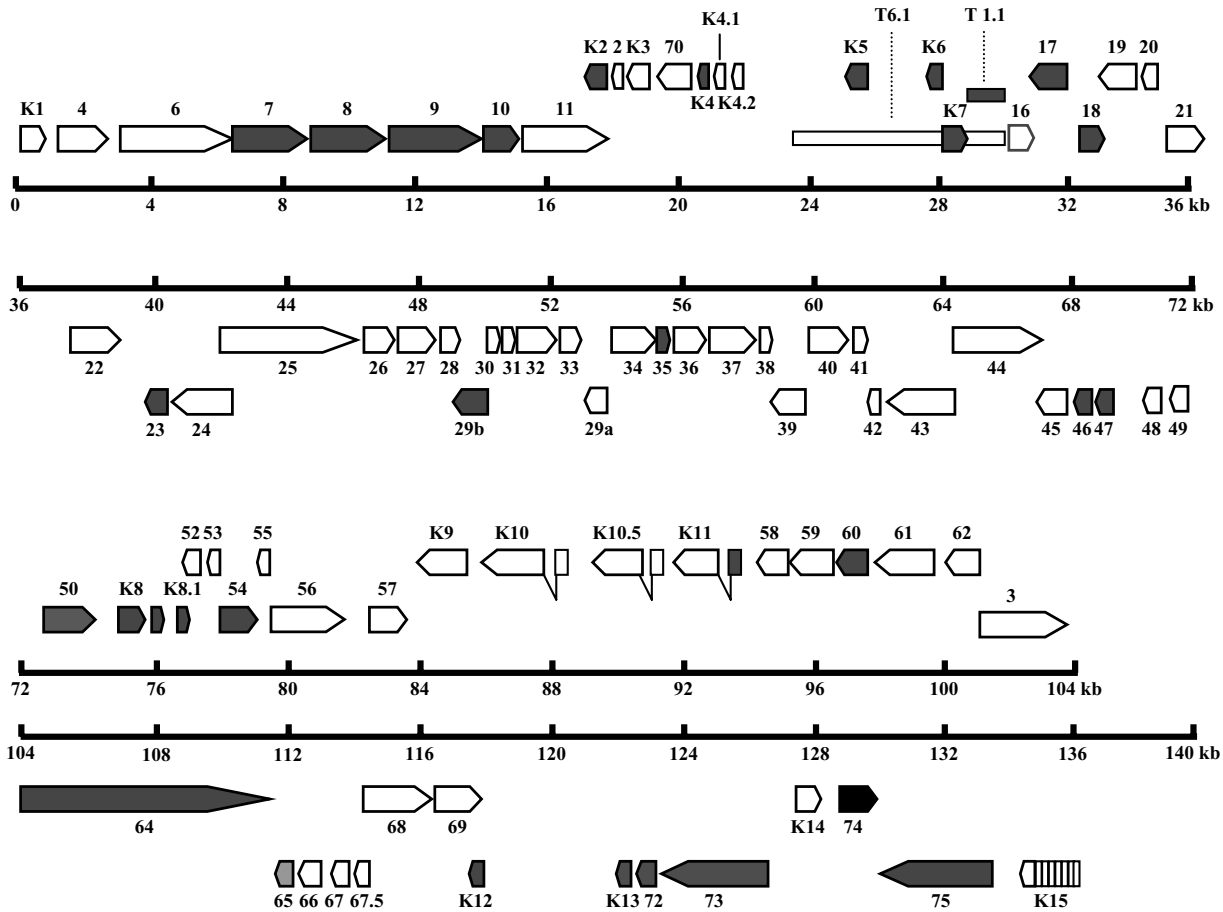

Fig. 28.3. KSHV gene expression pattern in endothelial and fibroblast cells following *de novo* infection. This figure summarizes the expression of individual KSHV genes following *de novo* infection of endothelial and fibroblast cultures reported in Ciufo *et al.*, 2001; Dezube *et al.*, 2002; Bechtel *et al.*, 2003; Gao *et al.*, 2003; Krishnan *et al.*, 2004.

- Latent gene
- Immediate-early gene in Krishnan *et al.* (2004)
- Transient expression during the first days after de novo infection (Dezube *et al.*, 2002; Ciufo *et al.*, 2001; Bechtel *et al.*, 2003; Krishnan *et al.*, 2004)
- Transient expression during first week of culture (Gao *et al.*, 2003)
- Cyclic expression pattern in the study by Dezube *et al.* (2002)

Initial results from immunohistochemistry suggest that some KSHV genes can become dysregulated in tissue culture and that tissue-specific patterns of expression exist. The lack of a KSHV-infected spindle cell line raises additional concerns regarding generalizing findings from infected B cell lines to endothelial-derived KS lesions.

KS lesions

The first studies on viral expression in KS lesions examined the abundance of the T1.1/PAN and T0.7/Kaposin transcripts by *in situ* hybridization. The vast majority of spindle cells comprising KS lesions expressed T0.7/Kaposin in a cytoplasmic distribution with a predicted membrane proclivity (Zhong *et al.*, 1996). By colocalization, a subpopulation (1%–10%) of T0.7/Kaposin positive cells also expressed T1.1/PAN transcripts and is thought to represent cells supporting lytic viral replication. The T1.1/PAN transcripts, although expressed in few cells, were present in high abundance ranging from an estimated 10 000 to 25 000 transcript copies per cell and were targeted to the nuclear compartment (Zhong *et al.*, 1996). Consistent with a transcript expressed in lytic replication, T1.1/PAN also co-localized to the same cell population with probes to

the major capsid protein (ORF25) (Staskus *et al.*, 1997) and to the viral GPCR (ORF74) (Kirschner *et al.*, 1999). The transcripts of two other genes, K3, K8 were shown to have a similar distribution to T1.1/Kaposin in KS lesions (Rimessi *et al.*, 2001).

By immunohistochemistry, LANA1 protein is expressed in almost all tumor spindle and endothelial cells (Rainbow *et al.*, 1997; Dupin *et al.*, 1999; Katano *et al.*, 2000). This is consistent with in vivo hybridization studies using the T0.7/Kaposin riboprobe as a surrogate marker for viral latent replication. Although vCYC and vFLIP proteins have not been formally shown to be expressed in a similar manner as LANA1, *in situ* hybridization studies of their transcripts in KS lesions demonstrate their presence in the majority of spindle cells (Reed *et al.*, 1998; Dittmer *et al.*, 1998; Sturzl *et al.*,1999). Reed and colleagues additionally found vCYC transcripts in epithelial cells of eccrine ducts and scattered epidermal cells (Reed *et al.*, 1998). In contrast to the expression of latent genes, only a few cells within KS tumors express proteins associated with lytic replication indicating a relatively tight regulation of lytic viral reactivation. The low number of cells hosting viral lytic reactivation in KS lesions is reflected in the numerous reports where lytic viral proteins are detected not at all or in only a few cells (<1%) when a series of KS lesions are examined. Viral proteins found to be expressing in rare cells of KS lesions include PF-8 (ORF59), (Katano *et al.*, 1999a,b; Parravicini *et al.*, 2000) and ORF50 (Katano *et al.*, 2001). The expression of vIL6 protein was found to be highly variable. Parravicini and colleagues were unable to detect the protein in 15 KS lesions they examined, while Cannon and colleagues found its expression in one out of 13 KS cases in one series but 7 out of 7 KS cases in another series deliberately selected for the presence of lytic foci (Parravicini *et al.*, 2000; Cannon *et al.*, 1999). The vIRF1 protein has not been detected in KS lesions by immunohistocheminstry thus far (Parravicini *et al.*, 2000). This is most likely due to antibody sensitivity, since cells hosting lytic replication would be expected to express the full spectrum of encoded proteins.

Primary effusion lymphomas

Due to the scarcity of these lymphomas, extensive sampling of *in vivo* viral gene expression has not been done in this disorder. In a pattern similar to that found in KS lesions, tumor cells in PEL all express LANA1 protein (Dupin *et al.*, 1999; Katano *et al.*, 1999a,b) and only rarely express ORF50 (Katano *et al.*, 2001). The major difference detected between PEL and KS lesions at this point is the expression of LANA2 which appears to be lymphoid specific. In contrast to ORF50 protein which is rarely detected, vIL6 is present in up to 5% of the PEL tumor cells. This partial uncoupling of vIL6 gene expression from RTA activation has been confirmed by in vitro studies on PEL cell lines showing that the vIL6 gene has a promoter containing two interferon stimulated response elements (ISRE) with the ability to induce vIL6 expression in cells treated with IFN-α. Additional evidence that transcriptional regulation of vIL6 is unique comes from microarray studies showing that only vIL6 transcripts in PEL cell lines were upregulated in response to IFN-α in the presence of cycloheximide (Chatterjee *et al.*, 2002). These results show that additional pathways, beyond the dichotomous latency-lytic pathways traditionally described for herpesviruses, regulate vIL6 transcription.

The LANA2/vIRF3 gene (K10.5) is one of the few KSHV proteins which has been found to be latently expressed in PEL and MCD cells in vivo; however, LANA2/vIRF3 is not expressed in the vast majority of KS spindle cells. This finding reinforces the concept that KSHV is capable of multiple latency expression programs, and genes that are expressed in some tissues or cell lines may be silenced or absent in others. LANA2/vIRF3 differs from vIL6 in that vIL6 is expressed in a minority population of PEL tumor cells. Since vIL6 is a secreted cytokine, limited expression of vIL6 may nonetheless contribute to the pathogenesis of PEL tumors. In contrast, LANA2/vIRF3 expression is uniformly present in PEL tumor cells, indicating that it may have a critical role in maintaining the PEL tumor cell phenotype. These patterns of expression could be expected if the vIL6 promoter is activated by cytokine signaling pathways that are dependent on the local cellular milieu, whereas the LANA2/vIRF3 promoter is activated by B-cell transcription factors. It bears repeating at this point that although PEL-derived cell lines also express LANA2/vIRF3 and vIL6 there is a greater percentage of cells positive for class II and class III proteins (see above) in vitro suggesting that regulation of KSHV protein expression may be different in culture (Parravicini *et al.*, 2000).

Multicentric Castleman's disease

In MCD, the majority of cells in affected lymphoid tissues are not infected with KSHV: the LANA1 positive cells are largely confined to the mantle zone of lymphoid follicles. These KSHV-infected cells are negative for T-cell or monocytic markers and are felt to be B-cells, although only a minority express CD20 or CD79 B-cell markers. A subset of these LANA1 positive mantle zone cells also express vIL6, K8, K10, PF-8, and ORF65 proteins. However, in contrast to both PEL and KS lesions there are a higher percentage

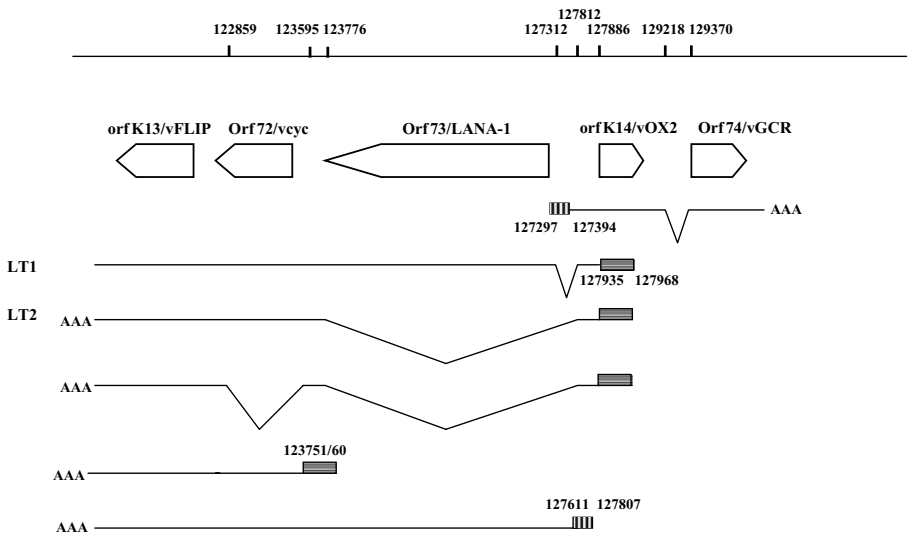

Fig. 28.4. Splicing pattern of latency-associated and neighboring transcripts. Two main transcripts, LT1 and LT2, and an additional minor transcript are directed by a latent promoter (horizontally striped box) and are translated to yield LANA1, vCYC and vFLIP. An additional latent promoter located in the 3′ end of the orf73/LANA1 gene (horizontally striped box) directs the expression of a bicistronic mRNA for vcyc and vFLIP. Both latent promoters also direct the expression of the kaposins and the viral miRNAs (see Fig. 29.8). In addition, expression of orf73/LANA1 can also be directed by a lytic promoter (vertically striped box). Overlapping with an intron spliced out of these transcripts is the promoter directing a bicistronic mRNA for ORFK14 and vGPCR. Numbering at the top refers to the position of splice donors and acceptors.

of LANA1 positive cells in MCD that express protein associated with lytic activation. Of these lytic cycle-associated genes, vIL6 appears to be expressed in a larger percentage of KSHV-infected cells. KSHV infected mantle zone cells therefore reproduce the pattern of viral gene expression observed in TPA-stimulated PEL-derived cell lines, in which a small, but significant subset of cells expresses class II and III genes (Parravicini *et al.*, 2000; Katano *et al.*, 2000).

Immunohistochemical techniques address critical aspects of virus behavior in infected tissue culture cells and pathological lesions that cannot be explored by mRNA studies. Although extensive mRNA mapping of viral gene expression has been performed in KSHV infected cell lines, tissue localization studies show that KS, PEL, and MCD are characterized by differing and unique patterns of KSHV protein expression.

Regulation of gene expression

Splicing

Genomic region containing KSHV latent genes and the viral chemokine receptor homologue

Gene expression in the locus encoding the major latent genes of KSHV, ORF73 (LANA1), ORF72 (vCYC), ORF71/K13 (vFLIP) is controlled by a constitutively active promoter located between nucleotides 127 935 and 129 370, with a minimal promoter region mapped to 127 935–127 968 of the prototypic KSHV sequence (Russo *et al.*, 1996), as shown in Fig. 28.4. (Jeong *et al.*, 2001). This promoter has the characteristics of a latent promoter, i.e. is not upregulated by treatment with phorbol esters or butyrate (Jeong *et al.*, 2004) however, it may be regulated in a cell cycle specific manner (Sarid *et al.*, 1999). It directs the expression of two more abundant and one rare mRNAs. The first transcript, latent transcript 1 (LT1; Fig. 28.4) encodes LANA1, while the second, LT2 represents a bicistronic mRNA from which both vCYC and vFLIP are translated. To enable efficient translation of the downstream reading frame for vFLIP, this mRNA contains an internal ribosomal entry site (IRES) located within ORF72 between nucleotides 122 973 and 123 206 (Bieleski & Talbot, 2001; Grundhoff & Ganem, 2001; Low *et al.*, 2001). In addition, Grundhoff & Ganem (2001) reported the existence of a further spliced mRNA from which most of the ORF72 coding sequence was removed and only vFLIP could be translated (see Fig. 28.4). However, this doubly spliced mRNA was of low abundance and only detected by RT-PCR after induction of the lytic cycle suggesting a mechanism for increasing the expression of this normally latent (Low *et al.*, 2001) anti-apoptotic

protein during lytic replication. Low *et al.* (2001) have also suggested that the IRES dependent translation of vFLIP may allow its expression during apoptosis when normal cap-dependent translation is less efficient due to cleavage of eIF4G by caspase 3 (Low *et al.*, 2001). A further bicistonic mRNA fo vcyc and vFLIP is directed by an additional latent promoter located in the 3′ end of the ORF73/LANA1 gene (Pearce *et al.*, 2005; Cai *et al.*, 2006). This promoter also directs the expression of a spliced mRNA, from which the Kaposin proteins are translated (see below and Fig. 28.8). In addition, un unspliced mRNA transcribed from his promoter serves as the precursor RNA for the viral miRNAs (see below and Fig 28.8).

Upregulation of LANA1 by the activator of the lytic cycle RTA shortly after infection of a cell by KSHV is directed by a lytic promoter (127,807–127,620) located downstream of the major constitutive (latent) LANA1 promoter. This lytic mRNA starts at position 127,611, i.e. within the intron in the LT1 latent mRNA (Matsumara et al., 2005).

A recent report (Canham and Talbot, 2004) suggests the existence of a further mRNA which is prematurely polyadenylated within ORF73 and would be predicted to encode a truncated variant of LANA1 lacking the 76 c-terminal amino acids. Based on a deletion analysis of the c-terminal end of LANA1 (Viejo-Borbolla *et al.*, 2003) such a truncated version of LANA1 would be expected to be deficient in its interaction with the nuclear matrix and in its ability to activate heterologous promoters and to replicate viral episomal DNA (Canham and Talbot, 2004; Viejo-Borbolla *et al.*, 2003).

The region upstream of ORF73 contains a second promoter that directs the expression of another bicistronic mRNA, expressed during the lytic replication cycle and oriented in the opposite direction (Fig. 28.4). This mRNA contains the reading frames K14 (vOX2) and 74 (vGPCR) (Talbot *et al.*, 1999; Kirshner *et al.*, 1999; Jeong *et al.*, 2001; Nador *et al.*, 2001; Chiou *et al.*, 2002; see Fig. 28.4). This promoter is activated by RTA, the central regulator of the lytic cycle and the minimal region response to RTA has been mapped to nucleotides 127 297–127 394 (Jeong *et al.*, 2004; Chiou *et al.*, 2002). At this promoter, activation by RTA is mediated through its interaction with the cellular transcriptional repressor RBP-Jκ, rather than by direct DNA binding (Liang and Ganem 2004). The bicistronic ORFK14/ORF74 mRNA results from splicing out an intron located between these two viral genes (Kirshner *et al.*, 1999; Talbot *et al.*, 1999; Nador *et al.*, 2001; Chiou *et al.*, 2002; Fig. 28.4). Although there is clear evidence from immunohistochemical staining of KS, PEL and MCD samples, as well as of TPA-induced PEL cell lines in vitro, that the vGPCR protein is expressed in cells undergoing lytic replication (Chiou *et al.*, 2002), it is not yet clear how this protein is translated from its downstream position in a bicistronic mRNA. Internal ribosomal entry, translational reinitiation, modified ribosomal scanning have been suggested as possible mechanisms (Kirshner *et al.*, 1999). In addition, Nador *et al.* (2001) found an additional monocistronic mRNA encoding only ORF74/vGPCR which was however much less abundant than the bicistronic mRNA for ORFs K14 and 74. The significance of this monocistronic mRNA and its contribution to vGPCR translation is not clear at present.

Genomic region containing immediate–early genes

The immediate–early gene ORF50, encoding the activator of the lytic cycle RTA, is located next to the multiply spliced early gene ORFK8, which encodes another player in lytic cycle regulation, KbZIP or RAP (Fig. 28.5). RTA is translated from an mRNA which incorporates a small exon located upstream of ORF49 in addition to the originally predicted reading frame 50 (Russo *et al.*, 1996; Lukac *et al.*, 1999; Zhu *et al.*, 1999; Saveliev *et al.*, 2002). The immediate–early expression kinetics of ORF50 mRNA, which has been reported to be upregulated by some groups as early as 1 hour following TPA treatment of PEL cell lines, has been discussed above. Expression of this mRNA is directed from a promoter that is autonomous and dependent on cellular factors (Seaman *et al.*, 1999; Deng *et al.*, 2000). In transient transfection assays using constructs in which the RTA promoter was placed upstream of a reporter gene, its activity can be further upregulated by RTA itself, but also by Na-butyrate or phorbol ester treatment and by co-transfection of some other viral proteins, e.g., vGPCR (Deng *et al.*, 2000; Chiou *et al.*, 2002). The autoactivation of the RTA promoter by RTA itself was also seen in persistently infected PEL cell lines (Deng *et al.*, 2000).

In latently infected cells the RTA promoter is partially silenced by methylation and it has been suggested that this provides a mechanism by which KSHV latency could be controlled (Chen *et al.*, 2001). Methylation of CpG islands in the RTA promoter region is seen in persistently infected PEL cell lines in vitro, as well as in samples from KSHV-associated diseases (KS, PEL, MCD) or KSHV-infected cells in vivo (Chen *et al.*, 2001). Treatment of PEL cell lines in vitro with 5-azacytidine, an inhibitor of methyltransferase, activates the lytic cycle (Chen *et al.*, 2001). Activation of the lytic cycle in PEL cell lines by TPA involves demethylation of the ORF50 promoter (Chen *et al.*, 2001). It has therefore been suggested that methylation of the ORF50 promoter is one of the mechanisms by which KSHV establishes latency in vivo (Chen *et al.*, 2001).

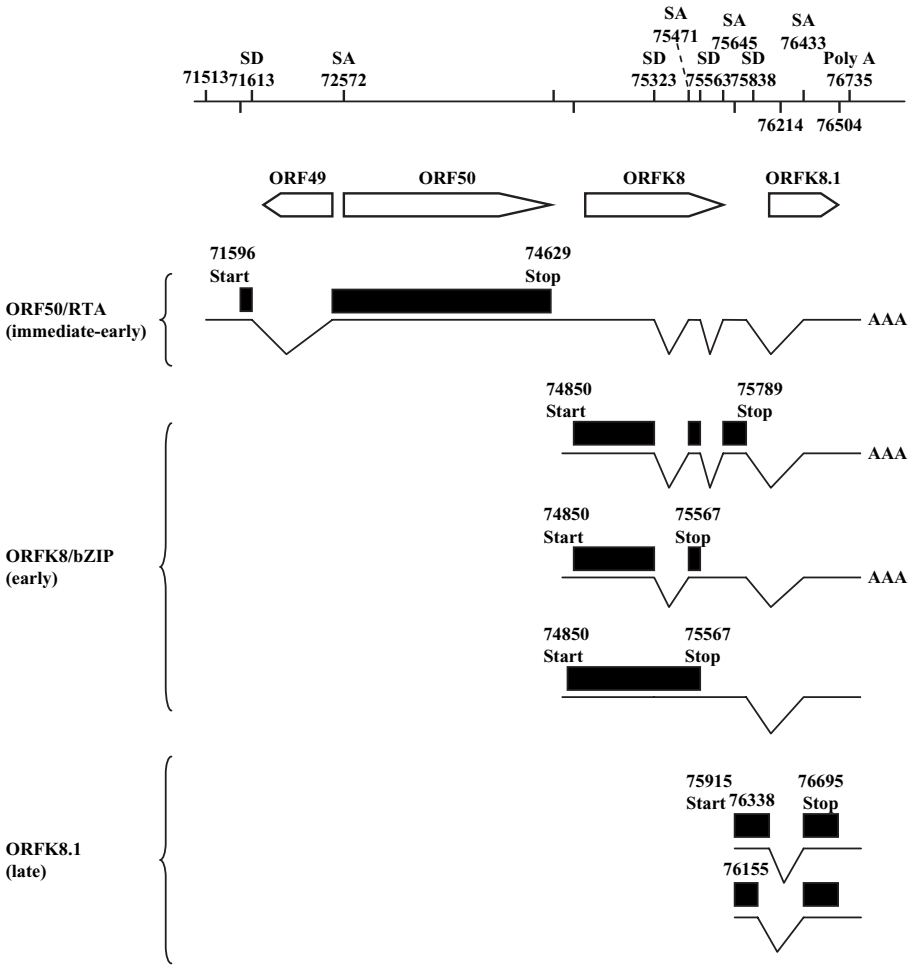

Fig. 28.5. Splicing pattern of transcripts originating in the immediate-early region of the KSHV genome. The region between ORFs 48 and 52 (see Fig. 28.1) encodes the immediate-early ORF50 transcript, as well as an early transcript for the ORFK8-derived proteins. The KbZIP protein involved in lytic replication consists of sequences included in 2 exons within the originally annotated ORFK8 in addition to a third, downstream exon encoding the leucine zipper region defining KbZIP. The mRNA for the ORFK8.1 glycoprotein also exists in two splice variants and has late expression kinetics (see Fig. 28.1).

The mRNA encoding ORF50 extends through two neighboring downstream genes, K8 and K8.1 (Fig. 28.5) but it is not clear whether there is any translation of these genes from this mRNA. However, a promoter located between ORF50 and K8 directs the expression of three alternatively spliced mRNAs, expressed at the ratio of 16:4:1 (Lin *et al.*, 1999; Fig. 28.5). One of these contains three splice events and consequently joins two exons from the originally predicted K8 reading frame to a downstream third exon which contains a leucine zipper region (Lin *et al.*, 1999; Gruffat *et al.*, 1999). Together with a basic region found in the second exon (Fig. 28.5) the resulting protein resembles members of the basic leucine-zipper transcription factors (bZIP) which includes jun, fos and the activator of the EBV lytic cycle, Zta or BZLF-1 (Sun *et al.*, 1998; Lin *et al.*, 1999; Gruffat *et al.*, 1999; Zhu *et al.*, 1999; Seaman *et al.*, 1999; further references in Sinclair, 2003). This protein, KbZIP or RAP is the predominant protein expressed in PEL cell lines after induction of the lytic cycle (Polson *et al.*, 2001). In contrast to Zta, KbZIP cannot on its own activate the lytic cycle (Lukac *et al.*, 1999; Polson *et al.*, 2001). On the contrary, it has been suggested that KbZIP, by binding to RTA, could repress the ability of RTA to activate some (e.g., ORF57), but not all (e.g., PAN/nut-1), lytic cycle genes (Izumiya *et al.*,

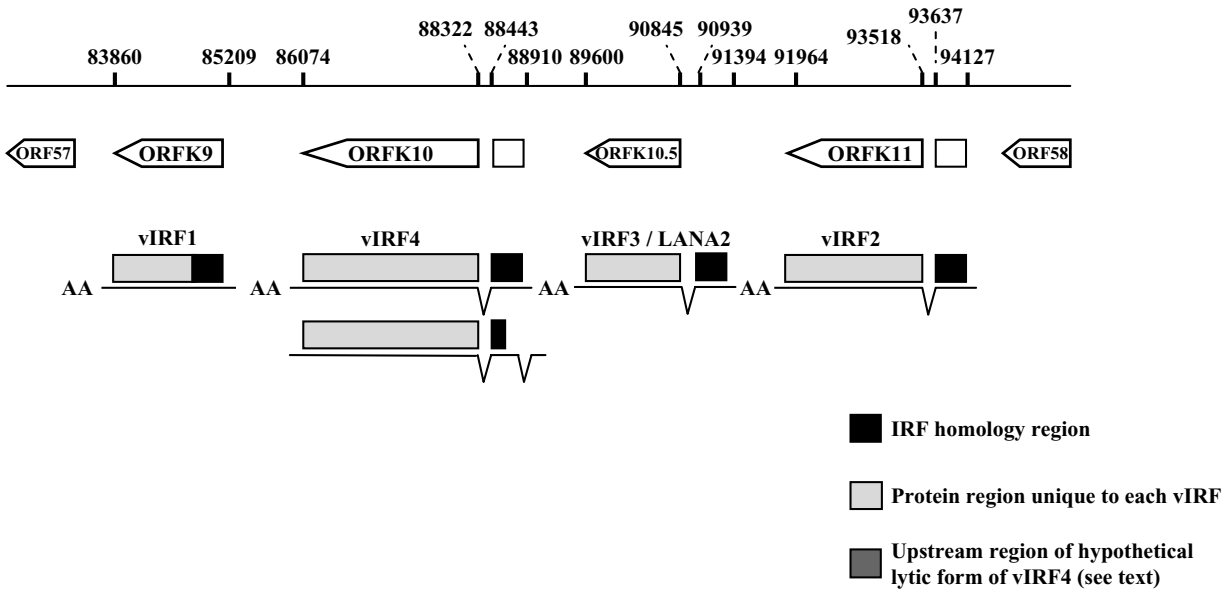

Fig. 28.6. Splicing patterns in the vIRF region of the viral genome. There are 4 KSHV proteins with sequence homology to cellular interferon regulatory factors (IRFs). The region of IRF homology is located at the amino terminal end of the viral proteins and encoded by a different exon in 3 out of 4 vIRFs.

2003a). The basic region of KbZIP is required for the interaction with RTA (Izumiya *et al.*, 2003a), as well as for the more recently described binding to cyclin A/E-cdk2 and its consequent phosphorylation by cdks (Polson *et al.*, 2001) and the resulting slowing of the cell cycle (Izuimya *et al.*, 2003b). The expression kinetics of KbZIP indicates that it is an early gene, since the corresponding mRNAs are sensitive to treatment with cycloheximide, but not phosphonoacetic acid (Lin *et al.*, 1999).

The fourth exon of the three K8/KbZIP mRNAs (Fig. 28.5) overlaps with another reading frame, ORFK8.1, encoding a structural glycoprotein incorporated into the KSHV virion and of importance for the binding of virions to glycosaminoglycans (Birkmann *et al.*, 2001; Wang *et al.*, 2001a, b). However, as shown in Fig. 28.5, this protein is translated from two alternatively spliced mRNAs, whose expression is controlled by a different promoter and which use the same splice acceptor at nt 76,433 as the K8/KbZIP mRNAs, but two different splice donors at nt 76,155 and nt 76,338 (Gruffat *et al.*, 1999; Raab *et al.*, 1998; Chandran *et al.*, 1998). This results in two isoforms of the K8.1 glycoprotein which run at molecular weights of 35 and 38 kDa on SDS gels (Raab *et al.*, 1998; Chandran *et al.*, 1998; Birkmann *et al.*, 2001; Wang *et al.*, 2001). As befits a structural virion glycoprotein, the K8.1 mRNAs have late expression kinetics, with doubling of expression between 16 and 24 hrs after treatment of PEL cell lines with TPA or overexpression of RTA (Nakamura *et al.*, 2003; Jenner *et al.*, 2001; Gradoville *et al.*, 2000) and sensitivity of the 0.9 kb K8.1 transcript to PAA (Gradoville *et al.*, 2000).

vIRF Locus

A gene locus located between nt 83,500 and 94,200 of the KSHV genome contains several homologues of cellular interferon regulatory factors, termed vIRFs (Russo *et al.*, 1996; Neipel *et al.*, 1997). Although initially controversial, the gene expression and splicing patterns, as well as the proteins encoded by the different ORFs in this locus, have recently become clearer. A current consensus view is summarized in Fig. 28.6 (Cunningham *et al.*, 2003). The first vIRF to be studied in detail, vIRF1 is encoded by an unspliced gene, ORFK9 (Gao *et al.*, 1997; Chen *et al.*, 2000; Wang *et al.*, 2001a, b; Cunningham *et al.*, 2003). ORFK9 shows expression kinetics compatible with its classification as an early gene: on Northern blots its expression is detected 8–12 hours after induction of PEL cell lines by TPA and is sensitive to treatment with cycloheximide but resistant to PAA (Wang *et al.*, 2001a, b). In DNA array studies ORFK9 expression peaked at 24 hours (Paulose-Murphy *et al.*, 2001; Nakamura *et al.*, 2003) and was classified as a secondary lytic gene by Jenner *et al.* (2001). Immunofluorescence studies with an antibody to vIRF1 have also indicated a marked increase in vIRF1 expression following TPA stimulation of PEL cell lines (Parravicini *et al.*, 2000).

In cells undergoing lytic viral replication a major transcriptional start site has been mapped to two adjacent

nucleotides 74 and 77 bp upstream of the translational start codon by one group (Chen *et al.*, 2001; Inagi *et al.*, 1999) and to a neighboring nucleotide (–76 bp) by two other groups (Wang *et al.*, 2001a, b; Cunningham *et al.*, 2003). This start site is located 20–25 bp downstream of a TATA box. In addition, Chen *et al.* (2001) reported another transcriptional start site, found only in latently infected cells, approx. 84 bp upstream of the lytic start site. In contrast, in spite of finding 5′ RACE products with varying start sites upstream of position – 77, Cunningham *et al.* (Cunningham *et al.*, 2003) could not confirm the existence of a defined latent start site and the evidence for the existence of an additional latent promoter for vIRF1 must therefore be seen as inconclusive. In vivo, vIRF1 expression could only be seen by immunohistochemistry in MCD tissue, but not in KS or PEL tissues, in keeping with the notion, derived from the in vitro studies, that vIRF-1 is expressed early during the lytic cycle (Parravicini *et al.*, 2000).

The neighboring viral gene, ORFK10, is also inducible in PEL cell lines (Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001; Nakamura *et al.*, 2003; Cunningham *et al.*, 2003). Jenner *et al.* and Nakamura *et al.* noted the very rapid onset of expression following chemical induction of PEL cell lines and Jenner *et al.* classified ORFK10 as a "latent/lytic" gene because of the basal expression seen in uninduced PEL cell lines. The ORF K10 transcript is spliced (Fig. 28.6). The open reading frame originally designated as K10 (Russo *et al.*, 1996) started at an ATG at position 88,164, i.e., within the larger downstream exon depicted in Fig. 28.6. For this reason, some authors refer to the smaller upstream exon as K10.1 (Jenner *et al.*, 2001; Neipel *et al.*, 1997). This upstream exon contains the protein regions with homology to the DNA binding domains of cellular IRFs (Jenner *et al.*, 2001; Cunningham *et al.*, 2003). However, analysis of the splicing patterns in the K10 region has shown that the original "K10" is expressed as part of a spliced mRNA that includes "K10.1" and consequently the inclusion of the IRF homology domains justifies the designation vIRF4 for the corresponding protein (Cunningham *et al.*, 2003; Jenner *et al.*, 2001; see Fig. 28.6). Cunningham *et al.* (2003) concluded that this spliced mRNA is inducible in PEL cells, while Jenner *et al.* (2001) considered this mRNA as "latent/lytic" but reported the existence of an additional alternatively spliced mRNA, found only in induced PEL cells, which eliminates the first 111 bp of the coding region in the upstream region. This would theoretically lead to a protein of 767 amino acids and a predicted molecular weight of 82 kDa, initiated at an internal ATG, which lacks the IRF homology region. However, Cunningham *et al.* (2003) found the alternative splice acceptor to be used by several mRNAs spliced to upstream viral regions as well as cellular sequences and queried whether this alternative transcript would be relevant physiologically. Using an antibody to K10, Katano *et al.* (2000) detected a dominant band of 100 kDa on Western Blots of induced but not uninduced PEL cell lines, in reasonable agreement with the predicted protein size of 98 kDa for the 905 aa translated from the singly spliced mRNA (Fig. 28.6). Although the existence of an additional minor protein cannot be completely ruled out, vIRF4, derived from the singly spliced mRNA, appears to be the dominant protein and expressed during the lytic cycle. By immunohistochemistry of pathology sections, vIRF4 was found to be expressed in 5% of KSHV-infected cells in MCD, but in less than 1% of cells in KS and PEL samples (Katano *et al.*, 2000). This staining pattern is compatible with the expression of K10/vIRF4 during lytic viral replication.

ORFK10.5 is contained in a spliced mRNA (Lubyova and Pitha, 2000; Rivas *et al.*, 2001; Jenner *et al.*, 2001; Cunningham *et al.*, 2003). In contrast to the original assignation of this ORF, the resulting protein (vIRF3, LANA2) contains sequences from both exons (Rivas *et al.*, 2001; Cunningham *et al.*, 2003; see Fig. 28.6). As in the case of the other vIRFs, a region with homology to cellular IRFs is located at the N-terminal end of vIRF3/LANA2 (Rivas *et al.*, 2001; Cunningham *et al.*, 2003; see Fig. 28.6) and derived from this first exon. While Lubyova and Pitha characterized this gene as inducible and Jenner *et al.* classified it as "secondary lytic" on their KSHV microarray using a probe for the upstream exon, Rivas *et al.* and Fakhari and Dittmer found it to be constitutively expressed in B-cells by northern blots or real time PCR. Using an antibody to vIRF3/LANA2, Rivas *et al.* could show its constitutive expression in latently infected PEL cell lines, as well as in PEL cell tumors and MCD specimens, but not in the endothelial and spindle cells of KS lesions. Cunningham *et al.* found several transcriptional start sites for the vIRF3/LANA2 transcript, none of which is in close proximity to a TATA box (Cunningham *et al.*, 2003). The TATA box noted by Lubyova and Pitha is located approximately 500 bp further upstream and thus unlikely to direct the transcriptional start of the vIRF3/LANA2 mRNA. However, Cunningham et al. noted the sequence element AAGGTAATGAGGT approx. 250 bp upstream of most 5′ RACE products in their study. This element is closely related to a motif AAGGTAATGAAAT in the latent LANA1 promoter (Talbot *et al.*, 1999) and the Oct-1/TAATGARAT element of immediate–early promoters in other herpesviruses (O'Hare, 1993).

ORFK11, as originally assigned by Russo *et al.* (1996), is also part of a larger coding region generated by a splice event that joins it to an upstream exon. As in the case of vIRF3/LANA2 and vIRF4, this upstream exon contains the region showing homology with cellular IRFs (Cunningham *et al.*, 2003). ORFK11/vIRF2 is an inducible gene in PEL cells (Sarid *et al.*, 1998; Cunningham *et al.*, 2003) which doubles

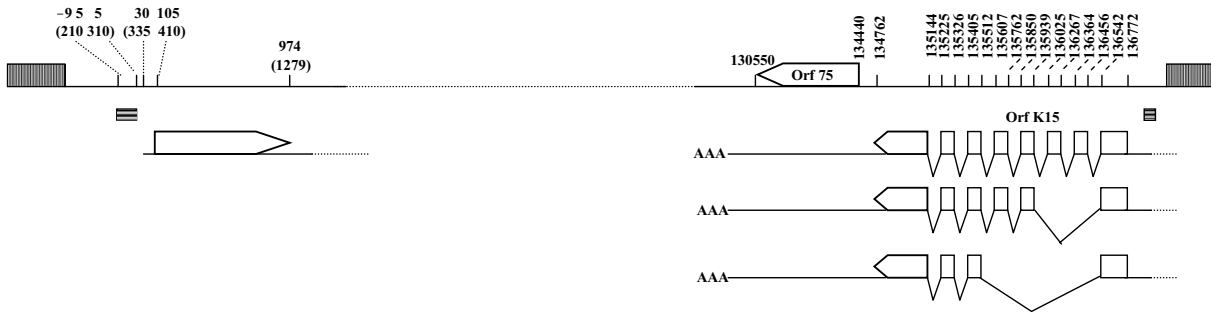

Fig. 28.7. Splicing pattern of transcripts and location of promoters at either end of the viral genome. The K1 gene at the "left" end of the viral genome is controlled by a promoter located next to the terminal repeat region. At the opposite end, the K15 gene is multiply and alternatively spliced to produce a group of proteins. A K15 promoter element is located adjacent to the terminal repeat region.

its basic expression level at about 20–24 hours after TPA treatment when measured on gene arrays (Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001). In induced PEL cell lines, the vIRF2 mRNA has a single transcriptional start site located 23 nucleotides downstream from a TATA box (Cunningham *et al.*, 2003). Using an antibody raised against ORFK11, Katano *et al.* (2000) could show that expression of the 110 kDa vIRF2 protein was only seen in TPA-induced PEL cells and that it is only rarely seen in KSHV-infected cells in KS, PEL or MCD, in keeping with its classification as a lytic gene product (Katano *et al.*, 2000).

Terminal membrane proteins

Two viral genes, K1 and K15, located at either end of the virus genome, encode membrane-associated proteins, VIP and TMP, respectively, that can trigger several cellular signal transduction pathways (Lee *et al.*, 1998a,b; Lee *et al.*, 2000, 2002; Lagunoff *et al.*, 1999, 2001; Glenn *et al.*, 1999; Poole *et al.*, 1999; Choi *et al.*, 2000; Brinkmann *et al.*, 2003). Both have no, or minimal, expression in uninduced PEL cells and mRNAs can be detected by northern blot, RT-PCR, RNAse protection or gene array following treatment with TPA or Na-butyrate (Lagunoff and Ganem, 1997; Sarid *et al.*, 1998; Glenn *et al.*, 1999; Choi *et al.*, 2000; Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001; Fakhari and Dittmer, 2002; Nakamura *et al.*, 2003). ORFK1 encodes a type I transmembrane protein, containing two hypervariable extracellular domains and an ITAM (immunoreceptor tyrosine activation motif) in its cytoplasmic domain (Lagunoff and Ganem, 1997; Lee *et al.*, 1998; Lagunoff *et al.*, 1999; Poole *et al.*, 1999; Cook *et al.*, 1999). The K1 encoded protein has therefore been termed VIP for variable ITAM containing protein.

ORFK1 gene expression was reported to increase significantly 8–10 hrs following TPA addition and to peak after 24 –72 hours. Lagunoff and Ganem (1997) found that the increase in its mRNA is not sensitive to PAA and therefore classified K1 as an early gene. The rate of increase of K1 gene expression led Jenner *et al.* (2001) to classify it as a 'tertiary lytic' gene, expressed with the same kinetics as many structural viral proteins. Similarly, Paulose-Murphy *et al.* (2001) placed it among the lytic genes with slightly delayed expression kinetics (peak expression after 36 hours). Nakamura *et al.* (2003) grouped K1 together with some structural proteins (ORF65/SCIP, a capsid protein; ORF47/gL, a virion glycoprotein; ORF62, a tegument gene), but also the ORF56 DNA replication protein and the ORF74/vGPCR chemokine receptor homologue.

The promoter for ORFK1 has been mapped to a region in the long unique region (LUR) of the viral genome immediately adjacent to the terminal repeats (Fig. 28.7). A 100 bp fragment corresponding to nucleotides 210–310 of the partial BCBL-1 sequence reported by Lagunoff and Ganem (1997), but located largely outside the prototypic KSHV genome sequence reported by Russo *et al.* (1996), can confer promoter activity to a heterologous indicator gene and has moderate but significant constitutive activity in B cells, epithelial cells and endothelial cells (Bowser *et al.*, 2002). The ORFK1 promoter is activated directly by RTA and TPA in B cells and epithelial cells; however, in SLK endothelial cells this effect is only weak (Bowser *et al.*, 2002). These results are in keeping with the reported lytic cycle expression kinetics of ORFK1. Lee *et al.* (2003) used monoclonal antibodies to the extracellular domain of K1 to demonstrate its expression early after induction of the lytic cycle in PEL cells and in a small proportion of KSHV-infected cells in MCD biopsies.

ORFK15, at the other end of the genome, consists of 8 exons, which are multiply and alternatively spliced (see Fig. 28.7) to give rise to a family of proteins that share a common c-terminal cytoplasmic domain encoded by exon 8 but vary in the number of membrane anchor domains

encoded by exons 1–7 (Glenn *et al.*, 1999; Poole *et al.*, 1999; Choi *et al.*, 2000). The reading frame originally designated as ORFK15 by Russo *et al.* (1996) represents only a small part of this gene and overlaps with exon 2 (see Fig. 28.7). The designation of TMP (for terminal membrane protein) has recently been adopted for the K15 proteins. The largest of the K15/TMP proteins has an apparent molecular weight of 45 kDa on SDS-PAGE, is predicted to contain 12 such transmembrane segments in addition to the c-terminal cytoplasmic domain, and has recently been shown to be a potent activator of the Ras/ERK, JNK and NF-κB pathways (Brinkmann *et al.*, 2003).

Like K1, K15 is inducible in PEL cells and has been classified as a class III gene on the basis of its inducibility but lack of basal expression in PEL cells (Sarid *et al.*, 1998). One of the three published gene array studies classified K15 as "tertiary lytic" (Jenner *et al.*, 2001), another (Nakamura *et al.*, 2003) reported a relatively early (8 hours) onset of K15 transcription which continued to increase up to 48 hours, whereas a third (Paulose-Murphy *et al.*, 2001) observed peak expression at 24 hours with a subsequent decrease. In all three gene array studies (Nakamura *et al.* 2003; Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001) K15 and K1 were grouped close to each other on cluster analysis. A promoter element directing the expression of the K15 gene has recently been identified in the long unique region between the first K15 exon and the terminal repeat region (Wong and Damania, 2006). A region derived from a terminal repeat subunit has promoter activity in vitro but is not responsive to RTA (Henke-Gendo, Rainbow & Schulz, unpublished data). In contrast to the similar expression kinetics of K1 and K15, regulation of K15 gene expression may therefore differ from that of K1.

At the protein level, different TMP isoforms have been demonstrated in transient transfection assays using expression constructs with differentially spliced mRNAs (Glenn *et al.*, 1999; Choi *et al.*, 2000; Sharp *et al.*, 2002; Brinkmann *et al.*, 2003). Recent findings suggest that the 45 kDa K15 protein is expressed in epithelial cell lines harboring a recombinant KSHV genome (M. M. Brinkmann *et al.*, unpublished data). A 21 kDa isoform, much smaller than the expected molecular weight of most TMPs, has been seen in some PEL cell lines and could represent a proteolytic cleavage fragment (Sharp *et al.*, 2002). Immunoreactive K15 protein has been detected in a small number of B cells in MCD biopsies using a monoclonal antibody to K15, in keeping with the predicted lytic expression pattern of K15 (Sharp *et al.*, 2002).

"Kaposin" locus

The region between nt 117,432 and nt 118,758 of the prototype KSHV sequence was originally predicted to contain an open reading frame, ORFK12, defined by a start ATG at position 117 919 and a stop codon at position 118 101 and expected to encode a small hydrophobic protein of 60 aa (Russo *et al.*, 1996; see Fig. 28.8). Independently, an 0.7 kb mRNA (T0.7) was cloned from a pulmonary KS sample and found to be latently expressed in KS biopsies and PEL cell lines by *in situ* hybridization (Zhong *et al.*, 1996; Staskus *et al.*, 1997; Stürzl *et al.*, 1997). By Northern blot, an mRNA originating in this region was also strongly expressed in uncultured PEL cells (Li *et al.*, 2002). K12/T0.7 has since been regarded as a marker of latently infected cells. However, in PEL cell lines, K12/T0.7 mRNA expression is induced by treatment with TPA or butyrate, with one group classifying it as a class II gene in view of its detectable basic expression in the BC-1 cell line in the absence of chemical treatment (Sarid *et al.*, 1998), while another classified it as an early gene because of the cycloheximide-sensitive induction of T0.7 mRNA 13 hours following Na-butyrate treatment of the same PEL cell line (Sun *et al.*, 1999). In more recent gene array experiments K12/T0.7 was classified as a latent/lytic gene (Jenner *et al.*, 2001) or as a lytic gene with relatively late doubling of expression (Paulose-Murphy *et al.*, 2001; Nakamura *et al.*, 2003). The contrast between the in vivo gene expression pattern (constitutive) and that in cultured PEL cell lines (inducible) suggests that the regulation of gene expression in PEL cells may have been affected by in vitro culture.

Although the term T0.7/Kaposin is now often used as a synonym for K12-derived transcripts, recent work has shown that in most PEL cell lines, in a primary PEL sample and in KS biopsies a larger transcript of varying size (approx. 1.4–2.4 kb, depending on the sample studied) predominates (Sadler *et al.*, 1999; Li *et al.*, 2002). The varying size of these transcripts is explained by the variable number of repeat units in two groups of repeats, DR1 and DR2 (Sadler *et al.*, 1999; Li *et al.*, 2002). This genomic arrangement has been seen in tumor samples or PEL lines of subtype A (see Fig. 28.8; Poole *et al.*, 1999). In addition to the variable length of the DR1 and DR2 repeats, additional repeat elements, I and IIa-c, have been described in a primary PEL sample of subtype B (Li *et al.*, 2002). However, the assignation of genomic subtypes in the K12 region is based on the sequence of the original K12 open reading frame (Fig. 28.8) and no extensive analysis of the genomic arrangement in the upstream repeat region in different KSHV subtypes has been carried out.

The larger mRNA encoded in this region originates at position 118,758 according to one report (Sadler *et al.*, 1999), or at position 123,842 between ORFs 72 (vCYC) and 73 (LANA1) and involving a splice event between nt 118,779 and 123,595 (Li *et al.*, 2002; see Fig. 28.8). The latter report found evidence for conservation of this splice

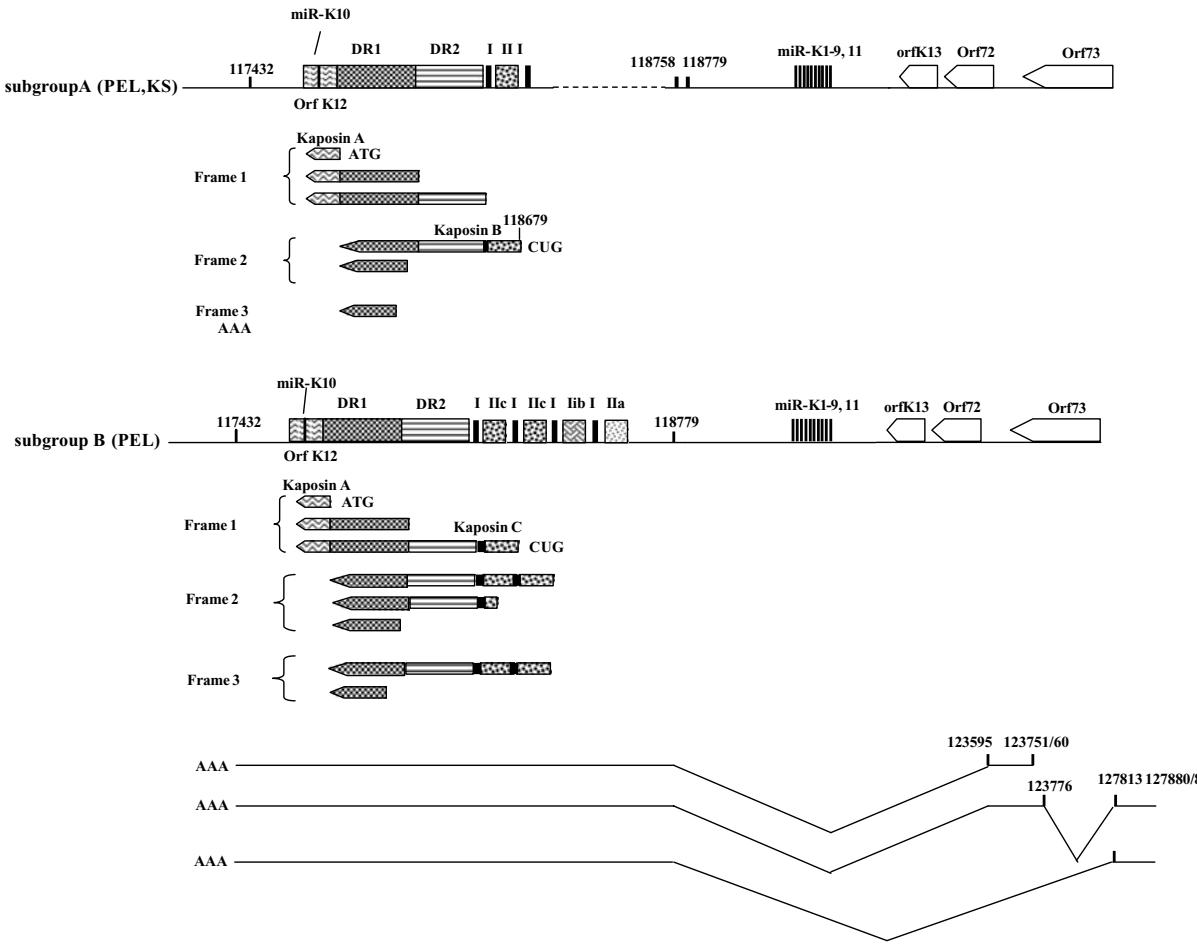

Fig. 28.8. Splicing patterns and translation products in the K12 region of the KSHV genome. Transcripts in the K12 region appear to originate upstream of K12. In the case of a primary PEL tumor, a promoter has been identified in the latent region of the genome, within ORF73. Translation appears to occur in different reading frames and from conventional (ATG) as well as unconventional (CTG) start codons.

event in all examined PEL cell lines and showed that the region between ORFs 72 and 73 contains a constitutive promoter element between nt 123,842 and 124,242, i.e., overlapping with ORF73 (LANA1) (Li *et al.*, 2002). The existence of this promoter has recently been confirmed by two other groups, although several start sites for the latent transcript originating at this promoter have been identified around 123751/60 (Pearce *et al.*, 2005; Cai *et al.*, 2006; see Fig. 28.8).

This latent mRNA uses alternative start codons (CUG, GUG) to translate several proteins in different frames whose sequence is derived from the repeats upstream of the original K12 (Sadler *et al.*, 1999; see Fig. 28.8). In frame 1 the original ORFK12 is also translated from a conventional ATG start codon, giving rise to the small (60 aa) hydrophobic membrane associated protein "kaposin A." Being thus located at the 3′ end of a bicistronic mRNA it is not clear at present whether its translation involves an internal ribosomal entry site, the inefficient use of the upstream alternative CUG start codon, ribosomal scanning, or a separate smaller mRNA. However, evidence for its expression in PEL cell lines has been presented (Kliche *et al.*, 2001).

A CUG start codon (nt 118 679) in frame 2 directs the expression of "kaposin B", a 48 kDa protein; evidence for its expression comes from transfection studies with an expression vector that contained an epitope tag in frame with, and downstream of, the predicted "kaposin B" sequence (Sadler *et al.*, 1999). "kaposin B" was the predominant protein expressed from the repeat region upstream of K12

(Sadler *et al.*, 1999) and also detected in a PEL cell line (Kliche *et al.*, 2001). However, the CUG start codon used by "kaposin B" was absent from the subtype B PEL sample investigated by Li *et al.* (2002). Not all KSHV subtypes may therefore express "kaposin B." A further alternative start codon (CUG) in frame 1 could be used to translate a third protein, "kaposin C"; however, expression of this protein appeared inefficient in the studies by Sadler *et al.* (1999) and Kliche *et al.* (2001). It is conceivable that it predominates in other KSHV subtypes (Li *et al.*, 2002), but no direct evidence for this currently exists.

As a consequence of the 23 bp repeat elements that constitute the DR1 and DR2 region the reading frame with regard to each individual element shifts in each consecutive element. The resulting proteins translated from the three different frames therefore share a repetitive 23 aa sequence motif (PGTWCPPPREPGALLPGNLVPSS for DR1; HPRNPARRTPGTRRGAPQEPGAA for DR2) and a monoclonal antibody to the DR1 motif will react with proteins translated in all three reading frames (Sadler *et al.*, 1999). This antibody detects DR1 containing proteins in a subpopulation of latently infected KS spindle cells, underscoring the expression of at least some DR1-derived proteins in vivo (Sadler *et al.*, 1999).

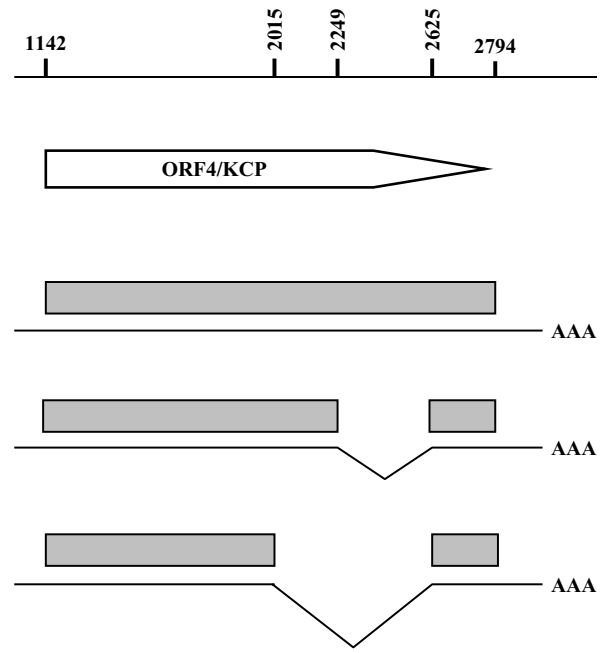

Fig. 28.9. Splicing patterns of ORF4 transcripts. Two of several alternatively spliced transcripts co-terminal with an unspliced full length transcript of ORF4, all inducible with TPA.

Viral microRNAs

In 2005, several groups independently identified a cluster of microRNAs (miRNAs) in the KSHV genome. Of 11 currently known miRNAs, 10 are encoded between the "Kaposin" locus and ORF71/K13 (miR K1-9,11), while a single miRNA has been identified in ORFK12 (Fig. 28.8). All KSHV miRNAs are derived from latent mRNAs directed by either the latency promoter in the 3′ end of ORF73/LANA1 or the major latency promoter upstream of ORF73/LANA1 (position 127,935–127,968; see Figs. 28.4 and 28.8). While miR K10 is located in an exon present in all spliced forms of these latent mRNAs the miR K1-9, 11 cluster is located in an intron present in the corresponding pre-mRNAs (Fig. 28.8; Cai *et al.*, 2005; Pfeffer *et al.*, 2005; Samols *et al.*, 2005; Grundhoff *et al.*, 2006). This ensures the expression of these miRNAs during latency, suggesting a role in the regulation of latent persistence. Potential viral and cellular mRNAs that could be targeted by these viral miRNAs are currently being explored.

Other spliced genes in the KSHV genome

ORF4 (KSHV complement control protein-KCP)

ORF4 encompasses nucleotides 1,142 through 1,794 and shares homology with cellular genes encoding proteins referred to as regulators of complement activation. Northern blot analysis and RT-PCR studies in PEL-derived cell lines demonstrate at least two alternatively spliced co-terminal transcripts in addition to an unspliced, full length mRNA of 1,679 bp (Fig. 28.9) which are induced by TPA treatment (Spiller *et al.*, 2003). These three transcripts encode proteins which retain C-terminal transmembrane domains and four N-terminal complement control protein (CCP) domains required for membrane attachment and complement regulation respectively. Analysis of complement regulation by soluble and membrane associated KCP demonstrated its ability to inhibit C3b deposition on cell surface and to act as a cofactor for factor I-mediated inactivation of complement proteins C3b and C4b, subunits of classical C3 convertase (Mullick *et al.*, 2003; Spiller *et al.*, 2003).

ORF40/41 (PAF)

ORF40 and ORF41 are both located on a spliced 2.2 kb mRNA that removes the region between these two ORFs and generates a long joint reading frame, ORF40/41, and has been shown to encode a protein of 75 kDa. Based on sequence homology, the 75 kDa protein is likely to be a primase-associated factor (PAF) (AuCoin and Pari, 2002; Wu *et al.*, 2001). The joint ORF40/41 transcript starts at position 60, 226, eliminates the genomic region between nt 61,658 and 61,784 and uses a polyadenylation site at position 62,546 (AuCoin and Pari, 2002; Wu *et al.*, 2001). A region

5′ to the start of this transcript has been found to contain a strong promoter activity when inserted into a luciferase vector (AuCoin and Pari, 2002). In addition, a second transcript of 0.7 kb initiates at nt 61,871 and thus only contains a part of ORF41; the corresponding protein is predicted to be translated from an ATG at position 61,908, within ORF41 (AuCoin and Pari, 2002). A 439 bp fragment upstream of the start site for this mRNA (nt 61,372–61,811) also has strong promoter activity (AuCoin and Pari, 2002). However, the existence of a protein translated from this mRNA has not yet been demonstrated. *ORF57*: The originally assigned ORF57 is located between nt 82,717 and 83,541 and was predicted to encode a homologue of an early herpesviral gene widely conserved among different herpesviruses, e.g., *herpesvirus saimiri* (HVS) ORF57, herpes simplex virus (HSV) ICP27 and Epstein–Barr Virus (EBV) BMLF1 (Russo *et al.*, 1996). For many of these homologues a role in RNA processing and nuclear export of unspliced mRNAs has been shown (detailed literature in Bello *et al.*, 1999). Subsequent RT-PCR studies, prompted by the smaller than expected size of KSHV ORF57, showed that the ORF57 mRNA is spliced and thus encodes a larger reading frame, of which the originally assigned ORF57 represents the c-terminal end. This splice removes an intron (nt 82,118–82,225) and with it an in frame stop codon upstream of the original ORF57. The spliced ORF57 mRNA initiates at nt 82,003, contains several in frame translational start codons in its first exon, uses the stop codon of the original ORF57 at 83,544 and a polyadenylation site at nt 83,608 (Bello *et al.*, 1999; Kirshner *et al.*, 2000). ORF57 is an early lytic gene whose expression becomes detectable on northern blots 2–4 hours after TPA induction of BCBL-1 cells, i.e., slightly later than ORF50/RTA and at about the same time as ORFK8/KbZIP, but before other early lytic genes (Lukac *et al.*, 1999). One of the more recent gene array studies classified it as a primary lytic gene (Jenner *et al.*, 2001), while another found a doubling of expression at 8h and a peak of expression at 72 h (Paulose-Murphy *et al.*, 2001). The ORF57 promoter is activated by RTA (Lukac *et al.*, 1999; Wang *et al.*, 2003a, b, c), placing ORF57 expression immediately downstream of the expression of RTA.

The ORF57 protein (SSM or MTA) enhances the expression of the bicistronic ORF59/ORF58 mRNA (as well as that of the ORF59/PFA protein), of the untranslated nuclear T1.1/PAN, RNA, and, in the presence of ORF50, RTA, of luciferase reporters driven by the nut-1 and kaposin promoters (Kirshner *et al.*, 1999). ORF57/MTA does not activate these promoters on its own, but enhances their ORF50/RTA-mediated activation. These findings suggest that ORF57 acts at a post-transcriptional level, but does so in a promoter-specific manner (Kirshner *et al.*, 2000).

ORFK3

The proteins encoded by ORFs K3 and K5 downmodulate major histocompatibility class I (MHC-I) proteins, NK receptors and coactivation molecules, thereby allowing KSHV-infected cells to escape both cytotoxic T-cell (CTL) and natural killer (NK) cell responses (Coscoy and Ganem, 2000; Haque *et al.*, 2000; Ishido *et al.*, 2000). Viral transcripts containing ORFK3 include three early (cycloheximide-sensitive) transcripts that also cover the neighboring ORF70 (TS). As shown in Fig. 28.10, one of these, an unspliced bicistronic mRNA, includes the entire ORF70 gene with the ORF70 translational start codon and presumably serves to translate the viral thymidylate synthase (Rimessi *et al.*, 2001). Another unspliced mRNA initiates downstream of the ORF70 start codon and could therefore translate a shortened ORF70 protein, or represent a monocistronic mRNA from which only the ORFK3 protein (MIR1, ZMP-B) could be translated. The third early mRNA splices out most of ORF70 and, although it does retain the ORF70 start codon and therefore represents a bicistronic transcript, could again serve to translate the ORFK3 protein. In addition to these early transcripts, an immediate-early, doubly spliced transcript that lacks most of the ORF70 coding region (but does retain the ORF70 ATG and is therefore bicistronic) could serve to translate the ORFK3 protein (Rimessi *et al.*, 2001).

Finally, a low abundance 2.5 kb latent transcript was recently identified by Taylor *et al.* (2005) which is coterminal with and encodes K3 in its entirety. When mapped, this transcript shows a complicated splicing pattern and encodes the entire K3 open reading frame (nt 18 585–19 671) along with 208 base pairs of the amino terminus of ORF70 (nt 20,096–20,304) and 70 base pairs of a region more than 3kb upstream (nt 23,770–23,840 (Fig. 28.10). Although low levels of ORFK3 protein expression has been documented in KS biopsies in vivo (Rimessi *et al.*, 2001), it is not yet clear which of these mRNAs is most efficiently translated to yield the ORFK3 protein and whether the novel 2.5kb latent transcript also plays a role in down-regulation of MHC class I during latent infection.

Events leading to the activation of immediate–early and early KSHV genes

In the experimental model commonly used to study the early stages of the lytic cycle, i.e., the induction of PEL cells by TPA or Na-butyrate, the immediate–early ORF50 gene, encoding RTA, is the earliest viral gene to be expressed. The ORF50/RTA promoter contains several transcription factorbinding sites, including AP-1, Sp1, Oct 1, CEBP/alpha (Deng *et al.*, 2000; Sakakibara *et al.*, 2001; Wang *et al.*,

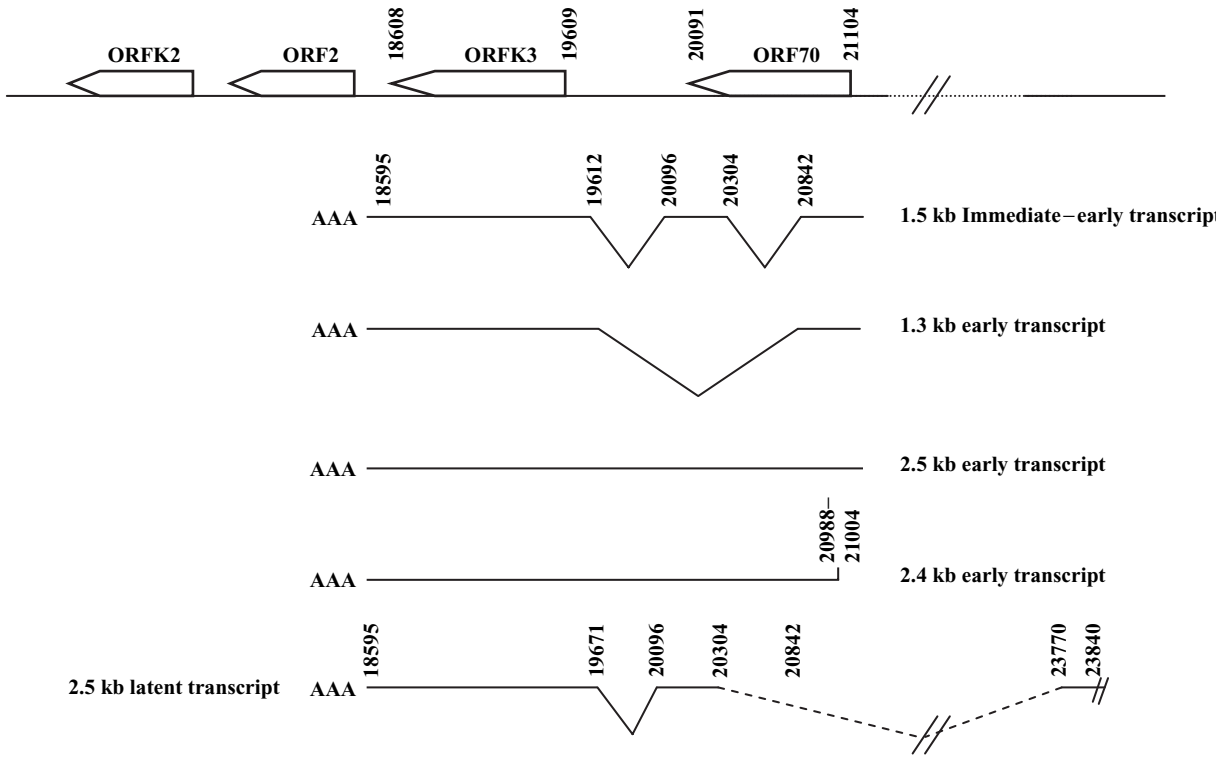

Fig. 28.10. Splicing patterns in the ORFK3-ORF70 region of the genome. Four transcripts are expressed during lytic induction of virus while a fifth transcript is detected at low levels during latent infection in PEL cell lines.

2003a,b,c). RTA activates its own promoter through two of three CEBP/alpha binding sites, most – likely by physically associating with CEBP/alpha (Wang *et al.*, 2003a,b). RTA also increases the expression of CEBP/alpha, thereby generating an amplification loop that leads to the expression of not only the immediate–early ORF50/RTA gene, but also of other early genes (see below).

In persistently infected PEL cell lines the ORF50/RTA promoter appears to be methylated in a region (–315 to –255 of the transcriptional start site) located between the two CEBP/alpha sites that are important for RTA-mediated activation (Chen *et al.*, 2001; Wang *et al.*, 2003a,b). In KSHV-infected B-cells in vivo this regions also appears to be heavily methylated, whereas in PEL biopsies, MCD lesions and in KS tumors in vivo, the methylation pattern is lighter, with an inverse correlation between the expression of lytic viral proteins and the methylation status of the ORF50 promoter in individual samples (Chen *et al.*, 2001). Treatment of persistently infected PEL cell lines with TPA or 5-azacytidine reduces the number of methylated CpG residues in the ORF50 promoter and activates the lytic cycle (Chen *et al.*, 2001). It is therefore, conceivable that methylation of the ORF50 promoter during viral persistence regulates, at least in part, the spontaneous activation of the lytic cycle in persistently infected cells. However, given the presence of transcription factor sites that are targeted by components of signaling pathways known to be induced by TPA or Na-butyrate (e.g., AP-1) these could also contribute to the activation of the lytic cycle.

RTA also activates a number of viral early promoters, including those of ORF, K8 (KbZIP/RAP), nut-1 (T1.1/PAN), ORF57 (MTA), ORFK2 (vIL6), vMIP, ORFK12 (kaposin), ORF74 (vOX2/GPCR). In at least some target promoters, i.e., T1.1/PAN, ORFK12, vIL6, RTA binds to specific DNA sequence elements (type II RTA responsive elements; RRE) (Chang *et al.*, 2002; Deng *et al.*, 2002; Song *et al.*, 2002; Wang *et al.*, 2003a,b). In contrast, the promoters for ORF50 (RTA), ORFK8 (KbZIP/RAP), nut-1 (PAN), and ORF57 (MTA) contain CEBP/alpha binding sites and their activation by RTA involves an interaction of RTA with DNA-bound CEBP/alpha (Wang *et al.*, 2003a,b).

In addition to the ability to directly bind DNA of target viral promoters, RTA can also target promoters lacking direct recognition elements by interaction with host DNA-binding factors such a RBP-Jκ. RBP-Jκ belongs to a family of sequence-specific transcriptional repressors which

recruits other corepressors to silence gene activation. By binding to RBP-Jκ, it appears that RTA not only displaces associated corepressors, but also allows for ligand independent activation of target genes. Although specific genes including PAN, ORF57, and SSB have been individually shown to be regulated in this manner, the broader implication is that RTA-mediated redirection of RBP-Jκ activity from repression to activation is critical for lytic reactivation (Liang *et al.*, 2002; Liang and Ganem, 2003).

The ORFK8 encoded protein, KbZIP or RAP, related to the EBV lytic-cycle Z transactivator (ZTA), as discussed above, contains a leucine zipper oligomerization domain and may interact with cellular transcription factors like CBP and CEBP/alpha (Wang *et al.*, 2003b). Although it plays a role in the KSHV lytic replication cycle, as shown by its association with PML domains and recruitment into lytic replication compartments (Wu *et al.*, 2001), it is, unlike EBV ZTA, not sufficient to trigger the activation of the lytic cycle and does not directly bind to viral DNA (Polson *et al.*, 2001; Chiou *et al.*, 2002). There is, however, evidence for its indirect association with the promoters for RTA, ORF57/MTA, as well as its own promoter, most likely as a consequence of its ability to associate with CEBP/alpha (Wang *et al.*, 2003b).

Regulation of the lytic cycle by other viral proteins

In addition to the regulatory role of RTA, KbZIP/RAP and ORF57/MTA during the immediate–early and early phase of the lytic cycle several other KSHV proteins may have the role of modulating lytic replication. Thus the membrane-associated glycoprotein encoded by ORFK1 has been found to inhibit the TPA-induced activation of the lytic cycle in the BCBL-1 PEL cell line (Lee *et al.*, 2002). A detailed analysis of the viral gene expression pattern in this cell line following the overexpression of K1 and treatment with TPA showed that the majority of viral genes appears to be downregulated as the result of K1 overexpression; however, a small number, including ORF72/vCYC, K15/TMP, ORF48 and K1 itself appeared to be upregulated. In contrast, K1 does not appear to affect the ORF50/RTA mediated activation of the lytic cycle, suggesting that TPA-induced events upstream of the ORF50/RTA promoter are modulated by K1. Among these the TPA-mediated activation of AP-1, NF-κB and Oct-1 in this PEL cell line appears to be inhibited by overexpression of K1 (Lee *et al.*, 2002). Lagunoff *et al.* (2001) also reported that K1 may moderately augment the activation of the lytic replication cycle in a PEL cell line but did observe a dominant negative effect of K1 signaling defective mutants on the ORF50/RTA-mediated activation of the lytic replication cycle in the same PEL cell line (BCBL-1).

Other factors that increase lytic viral replication

Several clinical observations suggest that reactivation of KSHV could be mediated by environmental factors or injury. Thus the frequent localization of classic KS lesions on the feet has been linked to an exposure to volcanic soil (Ziegler, 1993) or to reduced blood flow and poor oxygenation of the lower extremities in elderly individuals. Experimentally, hypoxia has been shown to activate KSHV lytic replication in PEL cell lines (Davis *et al.*, 2001). Haque *et al.* (2003) reported the presence of functional hypoxia response elements in the ORF50/RTA and ORF34 promoter. These elements are activated by either HIF-2 alpha (ORF50/RTA promoter) or by both HIF-1alpha and HIF-2alpha (ORF34 promoter) and hypoxia induces the transcription of ORF34 and ORF50/RTA mRNAs (Haque *et al.*, 2001). Chang *et al.* (2000) and Zoeteweij et al. (2001) reported that calcium ionophores, such as ionomycin and thapsigargin, could activate KSHV lytic replication cycle in PEL cells and further synergized with the effects of phorbol esters (Chang *et al.*, 2000). That KS lesions can arise in scar tissue or regions of traumatized skin (Köbner phenomenon) is another well-established clinical observation (Sachsenberg-Struder *et al.*, 1999).

In addition, an extensive body of experimental work has suggested some inflammatory cytokines may accelerate the development of KS lesions in AIDS patients (for a review see Ensoli *et al.*, 2001). Thus Monini *et al.* (1999) showed that inflammatory cytokines, and in particular interferon gamma, can increase the viral load in cultured PBMC of KSHV infected individuals. In a similar experiment Mercader *et al.* (2000) identified oncostatin M, hepatocyte growth factor/scatter factor and interferon gamma as cytokines that are released from HIV-1 infected T cells and can induce the expression of ORFK12 and ORF26 mRNA, as well as ORF59 and K8.1 proteins, in the BCBL-1 cell line. In vitro examination of KSHV infected cell lines demonstrates that inflammatory cytokines had diverse effects on KSHV induction. While interferon gamma consistently induced lytic activation, Chang *et al.* (2000) found other cytokines including tumor necrosis factor, IL-1, IL-2, IL-6, granulocyte-macrophage colony stimulating factor, and basic fibroblast growth factor did not. Further, interferon alpha inhibited KSHV induction (Monini *et al.*, 1999; Chang *et al.*, 2000).

Recently, it was shown that KSHV-infected keratinocytes could activate the lytic replication cycle upon epithelial differentiation in raft cultures (Johnson *et al.*, 2005), suggesting that, similar to human papillomavirus, KSHV infected epithelial cells could be programmed to allow the production of new virions at the epithelial surface.

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29

Effects on apoptosis, cell cycle and transformation, and comparative aspects of EBV with other known DNA tumor viruses

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The list of human viruses presently known to cause or to contribute to tumor development comprise four DNA viruses, Epstein–Barr virus, certain human papilloma virus subtypes, hepatitis B virus, and Kaposi sarcoma herpesvirus (HHV-8); and two RNA viruses, adult T-cell leukemia virus (HTLV-1) and hepatitis virus C. In addition, while HIV infection is not directly tumorigenic, it increases the incidence of certain tumors.

The purpose of this chapter is to consider EBV and HHV-8 in relation to the known DNA tumor viruses, with particular focus on tumorigenicity.

Viral strategy at the molecular level as a tumor risk factor

Altered genes or environmental factors are usually considered as major risk factors for tumor development. However, the strategy of certain viruses may constitute a risk factor in itself. Tumor-associated viruses in humans have a survival strategy, like other viruses, aiming to maintain, replicate and propagate their genomes, but some features of this strategy entail a risk to initiate or favor tumor development under certain circumstances. This implies that only a small minority of the infected cells enter the pathway towards a malignant tumor and even fewer succeed.

Three types of virus–host cell interactions may carry a risk

1. Blocking of late viral functions or blocking the replicative cycle, by mutation or deletion of genetic material, e.g., due to the integration of the viral genome, as exemplified by HPV or adenovirus transformation in vitro.
2. Infection of cells that are not fully permissive for viral replication, for species or tissue specific reasons. Permissiveness for the early but not the late functions of the viral cycle is particularly dangerous. The early viral proteins may exert continuous proliferation stimulating and/or apoptosis preventing effects. Infection of hamster or guinea pig cells with some of the human adenoviruses and SV40 infection of rodent cells may serve as examples.
3. Latent viral persistence may subvert normal controls. This can be illustrated by EBV infection of B-lymphocyes in immunodefective hosts.

Early history: up and down

Views on the role of viruses in the etiology of cancer have been polarized between two extreme positions during the major part of the last century. The belief that viruses have nothing to do with cancer was as widespread at certain times, as the suspicion that most and perhaps all tumors are caused by virus at other times. The field started with the discovery of Peyton Rous in 1911 that chicken sarcomas could be transmitted with cell-free filtrates (Rous, 1911). The tumors arose at the site of inoculation and were of the same histological type as the original sarcoma. This created great excitement : the cancer problem was solved! The enthusiasm subsided rapidly, however, when mouse and rat tumor filtrates failed to induce tumors. In retrospect we may see this as the consequence of exaggerated expectations, hasty experiments and increasing lack of confidence. It became the prevalent view that viruses may play a role for tumors in birds, but not in mammals.

Two decades later, Richard Shope (1933) found that benign warts could be transmitted from the wild cottontail

to the domestic rabbit by cell-free filtrates. This did not change the climate of opinion. The rabbit was a mammalian but warts were benign tumors, not cancers. Several important points were overlooked by outside commentators, however. The initially benign rabbit papillomas turned occasionally into carcinomas. This could be accelerated by the topical application of chemical carcinogens. The term tumor progression was originally coined by Rous to designate this transition, or, in its generalized form, the process whereby "tumors go from bad to worse." Later, Leslie Foulds (1958) defined and extended the term. It refers to the development of tumors by multiple, stepwise changes in several "unit characteristics." Today we see them as distinct phenotypic traits. They are individually variable and reassort independently of each other. Tumor progression can therefore proceed along several alternative pathways and each tumor becomes individually unique from the biological point of view.

The early work on Shope papilloma was also interesting from the immunologist's point of view. The virally induced warts that did not progress to carcinoma were rejected simultaneously by a systemically acting host response, mediated by lymphocytes, rather than by antibodies. This was the first example of a tumor rejection response that targeted virally encoded proteins in DNA virus transformed cells.

In the 1930s, John Bittner (1936) discovered the milk factor, later called the mouse mammary tumor virus (MMTV). This discovery did not create any major change of opinion either. This may have been due, at least in part, to the way the findings were presented and discussed. The genetically oriented mouse mammary tumor biologists proceed by careful, gradual analysis that fitted the long duration of each experiment (2 years or more). It showed that MMTV could increase the frequency of mammary cancer, but it was neither necessary nor sufficient for tumor induction. Hormonal and genetic factors modified the risk considerably. The role of MMTV as a tumor-susceptibility factor in selectively inbred high cancer strains was readily accepted, but its role as a "tumor virus" remained questionable. It was appreciated, however, that the probability of tumor development could be influenced by multiple factors, including viruses.

Up again, and how!

A major paradigmatic shift occurred in the 1950s. It was triggered by the discovery of the murine leukemia virus by Ludwik Gross (1951) and the polyoma virus by Sarah Stewart and Bernice Eddy(Stewart *et al.*, 1958). Gross found that cell free filtrates prepared from the "spontaneous" leukemias of the high leukemic AKR strain could transmit the disease to a low leukemia strain, C3H. In contrast to many others who failed before him, Gross succeeded for three reasons: his serendipitous use of newborn, less than 24 hours old mice as recipients; his fortuitous choice of C3H, the only low leukemia strain available at the time that happened to be susceptible to the virus carried by the AKR strain, later called the Gross virus; and the dogged persistence of Ludvik Gross in an area where nobody expected positive results.

The scientific community received Gross's first report with surprise and disbelief. This attitude prevailed for 5 years, until the originator of the AKR strain, Jacob Furth, took pains to repeat Gross's experiments under the original conditions and with the same recipient C3H subline (Furth *et al.*, 1956). He succeeded, in contrast to others who were less meticulous in their choice of experimental conditions. Furth's confirmation has led to the immediate acceptance of Gross's findings. The discovery of the polyoma virus also stemmed from Gross's work, but in a more indirect fashion. Gross has observed occasional parotid tumors in C3H mice inoculated with AKR leukemia filtrates. He realized that they may have been induced by another virus, provisionally referred to as the parotid tumor agent.

Stewart and Eddy started out on the assumption that Gross's leukemia virus experiments were correct. Since the virus was apparently quite weak, however, they wished to amplify it by adding the leukemia filtrates to embryonic mouse fibroblast cultures. After a few days culturing, they inoculated the filtered supernatants into newborn mice. The mice developed a wide variety of sarcomas and carcinomas, but no leukemia. Due to its ability to induce many types of tumors, the virus was named polyoma.

Classes of experimental tumor viruses

The viruses so far mentioned fall into three major categories. Rous sarcoma virus belongs to the acute or class I RNA tumor viruses. The murine leukemia and the mammary tumor virus fall into the category of chronic or class II RNA tumor viruses. The Shope papilloma and the polyoma virus are DNA tumor viruses.

Some interesting generalizations can be made on the basis of this and later experimental work that has identified many additional viruses in all three categories.

All experimentally derived RNA tumor viruses belong to the retrovirus family. They carry their genetic information in RNA. Following their entry into a susceptible target cell, the virally encoded reverse transcriptase rewrites their RNA into proviral DNA that can insert into cellular

DNA at random. When virus production is activated again, the proviral DNA is transcribed into RNA. This is followed by viral RNA replication, the production of new viral proteins, the assembly of new viral particles, and their release by budding, but it is not accompanied by any cytopathic effect. Virus production is therefore compatible with cell proliferation.

Activation and transcription of the integrated provirus is an error-prone process. Adjacent cellular DNA may contribute to the RNA sequences carried by the viral particle. In the vast majority of the cases, this has no notable consequences, but occasionally the incorporated cellular sequence may originate from a gene whose activated product can stimulate the entry of the cell into the S-phase. Virus particles that carry such sequences may cause cell proliferation when they infect new recipient cells. The probability that this happens is very low, because every step in the process, from the integration of the virus into the "right place," through the production of the appropriately (in frame) fused viral–cellular messages, the release and the replication of competent virus and the subsequent new infection of a susceptible cell, are all low probability events. A tumorigenic virus variant is usually generated by the purposeful and often prolonged selection for tumorigenicity This requires great persistence on the part of the investigator. Following the early discovery of the Rous sarcoma virus, it took four decades before new acute or class I RNA tumor viruses were isolated that could induce tumors at the site of inoculation and to transform normal into tumor cells in vitro. Following the revival of viral oncology in the 1950s, some 40 such viral strains, carrying about 20 different cell derived oncogenes, as they were to be called, were isolated in rapid succession from fowl, rodent, feline and simian tumors.

Class I RNA tumor viruses are not known to play any tumorigenic role in nature. This is understandable, because most of them are defective, due to the replacement of essential viral genetic information, by the inserted cellular gene. With only some notable exceptions, they produce crippled virus particles that can only multiply in the presence of complete, but non-transforming "helper virus."

Chronic or class II RNA tumor viruses have no transforming activity in culture. They do not induce tumors at the site of inoculation and carry no cellular oncogenes. Insertion of the proviral DNA in the immediate neighborhood of a cellular oncogene is the most frequent mechanism whereby they contribute to the tumorigenic process. Since the proviral DNA integrates at random, the likelihood of such an insertion is low. Very high level of virus production, accompanied by viremia, is usually mandatory for tumorigenicity. This is the reason why only some mouse strains that can support virus replication and/or are deficient in their immunological responsiveness to the virus, are susceptible to the tumorigenic effect of murine leukemia virus or mammary tumor virus.

Insertion in the neighborhood of a cellular protooncogene is not the only mechanism whereby an RNA tumor virus can initiate tumor development, but other alternatives are less well documented. HTLV-1, or adult T cell leukemia (ATLV) virus, is an example of this. It is believed to stimulate the expansion of preneoplastic cell populations, paving the way to cellular changes that may be more directly involved in the tumorigenic process (Gallo *et al.*, 1983).

In conclusion, the RNA tumor viruses have provided a wealth of information about virus-cell interactions in relation to the tumorigenic process and have led, indirectly, to the discovery of numerous cell division regulating cellular genes. They can be regarded as a model of what can happen, but they give us very little information about what does actually happen in the genesis of human tumors.

The DNA tumor viruses provide a very different picture. They belong to several unrelated virus families. In contrast to the RNA tumor viruses that can replicate in growing cells without killing them, the DNA tumor viruses kill the cells in which they replicate. Their tumorigenic activity depends therefore on the blocking of the lytic viral cycle. This may occur in cells that are non-permissive for the lytic cycle due to their species and/or tissue derivation.

The transforming genes of all DNA tumor viruses are genuine constituents of the viral genome. The number of virally encoded transforming genes varies between one (SV40), two (adeno- and papillomaviruses) and six (EBV). The virally encoded transforming proteins are immunogenic, as a rule. The challenge of viral transformation is met by the immune surveillance of the host. Cells transformed by these viruses grow usually only in immunosuppressed hosts. They represent the major part of the "opportunistic tumors" that arise exclusively or predominantly in congenitally, iatrogenically (as after organ transplantation) or virally (e.g., by HIV) immunosuppressed persons.

Inactivation of Rb and p53 is an important prelude to viral replication, as discussed below. Early after primary infection, DNA tumor viruses induce a round of DNA replication in the recipient cells. This carries the risk of malignant proliferation. The host inhibits the progressive growth of the transformed cells by its immune response, however. While the immunocompetent host rejects virus driven, proliferating cells, the virus goes into hiding. It persists in non-proliferating cells where it is not "seen" by the immune response. The example of EBV provides a particularly interesting "success story" that favors the survival of both the

virus and the host. A comparison with the smaller DNA tumor viruses, in the next section highlights both parallels and contrasts.

What does the type of virus-cell interaction tell us about tumorigenic risk?

There is a fundamental difference between the small DNA tumor viruses and the herpesviruses, with regard to potentially tumorigenic interactions with their host cell. Permissiveness for the early but not for the late (lytic) steps of the viral cycle constitute the main risk for the former group. Convergent evolution has provided SV40, the transforming adenoviruses and the tumor associated papillomaviruses with the ability to inactivate two of the main tumor suppressor pathways that involve p53 and Rb, respectively. Inactivation of the same two pathways appears to be mandatory for non-virally related tumor development. As discussed elsewhere in this chapter, this impairs two of the main controlling functions that prevent the replication of cells driven by illegitimately activated oncogenes.

Inactivation of both pathways by the small DNA tumor viruses is part of the viral strategy. Both the integrating and the episomal viruses need to induce an S-phase, as already mentioned, in order to integrate or to establish the appropriate chromosomal–episomal balance, respectively. They also need to protect the activated cell from apoptosis, in order to secure their persistence.

In the natural host cell, the growth stimulating and anti-apoptotic effects of these viruses have no lasting consequence, because virus production and cell lysis sets a natural endpoint.

In the case of the tumor associated herpesviruses and particularly of EBV, the possible tumorigenic contributions of the virus need to be considered in relation to the different forms of non-productive virus–cell interactions. EBV has evolved mechanisms to activate and expand its primary host cell population, the human B-lymphocyte. It is also capable of switching off its B-cell activating program and remain latent in long-lived resting memory B cells. The virus can thus use several non-lytic interaction programs, tailored to different B cell subclasses. They lead to different programs of viral expression that also differ in their ability to induce a CD8+ T-cell mediated immune response in the host that prevents the excessive proliferation of the virally transformed immunoblasts.

Unlike the small DNA tumor viruses, where tumorigenicity is favored by the structural or regulatory impairment of the lytic genes, the latent EBV-B cell interaction that occurs without any genetic defect in the virus, is a potential tumor risk in the immunodeficient host. This is due to the fact that, apart from the occasional activation of the viral cycle in EBV-carrying B cells, the interaction is largely non-lytic. In EBV-carrying immunoblastomas that arise in transplant recipients, or in certain congenitally T-cell defective patients (XLP in particular) as well as in part of the AIDS associated lymphomas (with immunoblastic morphology), the interaction of the virus with proliferating immunoblasts is comparable to or identical with the usual interaction of the virus with normal B-cells. The tumorigenic "accident" occurs at the level of the host and not at the level of the virus or the cell.

None of the other EBV-associated B-cell derived malignant lymphomas, such as BL, HL, or PEL or the unusual, EBV carrying T-cell lymphomas express the proliferation driving, blastogenic program of the virus. The virus expresses only minimalistic programs designated as latency I or II, in contrast to the full immunoblastic program (III). The tumor promoting contribution of the virus must therefore be sought in other, less direct effects.

In BL, one needs to depart from the fact that the lymphoma originates in the post-GC, centroblastic or centrocytic cell, that is either resting or is on its way to a long-lived resting memory cell, but cannot leave the cycling compartment because it is driven by an Ig/myc translocation. The translocation results from a faulty recombination that occurs in the course of physiological Ig gene rearrangement. Conceivably, EBV may contribute to the emergence of the virus carrying BL clone by expanding the original population or, alternatively, or in addition, by protecting the myc-driven, apoptosis prone cell from apoptosis. (See further in the section on EBV and BL below).

Human tumor viruses

Four of the six viruses known to be involved in the causation of human cancer in a direct or a contributory capacity are DNA viruses (EBV, HPV, HBV, HHV-8) while the remaining two are RNA viruses (HTLV-1 and HCV).

As discussed below, EBV is most directly involved in the causation of immunoblastomas that arise in immunodefective persons, such as transplant recipients, certain congenital immunodefectives, and HIV-infected persons. EBV may also play a role in Burkitt lymphoma (BL) and nasopharyngeal carcinoma (NPC), as indicated by the regularity of its association with these tumors, but the nature of the viral contribution is not fully understood.

Special subtypes of the human papilloma viruses are known to contribute to the genesis of cervical carcinomas and of skin tumors. Human herpesvirus no.8 (HHV8, also called KSHV) is associated with Kaposi sarcoma,

Castelman's disease and body cavity lymphoma. Hepatitis virus type B and C contribute to the genesis of primary liver cancer. The evidence for these virus-tumor associations is epidemiological and molecular, but the relative role of the virus and of cellular changes has not been properly established.

The following two sections deal with the two human tumor associated gamma herpesviruses, Epstein–Barr virus (EBV) and, to a minor extent, Kaposi sarcoma herpesvirus (KSHV or HHV-8).

Epstein-barr virus (EBV)

EBV is the causative agent of a self-limiting lymphoproliferative disease, infectious mononucleosis. In immunodefectives, the proliferation may proceed to progressively growing immunoblastomas. Multiple viral genomes, derived from a single infectious event, are regularly found in high endemic Burkitt lymphomas (BLs) and in low differentiated or anaplastic nasopharyngeal carcinomas (NPCs). EBV is also found, although less regularly, in Hodgkin's lymphomas, nasal T-cell lymphomas, gastric carcinomas, salivary gland tumors and leiomyosarcomas (Table 29.1).

Table 29.1. EBV-associated tumors in man (the percentage figures indicate the frequency of EBV carrying tumors)

Lymphoid tissues		Epithelial tissues	
Burkitt's lymphoma, endemic	98%		
Burkitt's lymphoma, sporadic	25%	Gastric adenocarcinoma	5–10%
AIDS-immunoblastic lymphoma – in CNS	60% 100%	Nasopharyngeal carcinoma undifferentiated	100%
Post-transplant lymphoma	100%	Salivary gland carcinomas	<100%
Hodgkin's lymphoma	50%	Leiomyosarcoma in immunosuppressed	100%
T-cell lymphomas -lethal midline granuloma	10–30% >90%		

EBV exploits B-cell specific regulatory mechanisms and signals

Normal B cell physiology is tightly regulated. The generation of B-cell receptor diversity by immunoglobulin gene rearrangements, activation of resting B cells by cognate antigen and associated molecules, expansion of the activated population, migration through germinal centers and concomitant hypermutation, generation of long-lived memory cells and differentiation into secretory subtypes, plasma cells in particular, is regulated both by internal programs and external signals, including antigens, ligands and cytokines that influence homing, proliferation, differentiation and death of the cells. EBV has a whole gamut of highly refined mechanisms that exploit normal B cell physiology. Unlike many other viruses, its strategy is not limited to the turning of its host cell into a viral protein factory with the single purpose of virus production and release to the environment, although it can switch on that mechanism when it enters the lytic cycle. Rather, the virus follows a "live and let live" principle. Its vast success in infecting all human populations and persisting in latent form over the lifetime of the host without causing disease, except by accident, testifies to the validity of this strategy, both for the virus and for the cells.

The exploitation of normal B-cell physiology by the virus manifests itself at many different points. Already the very first step, viral attachment and penetration, is based on the use of a B-cell specific surface moiety, CD21 (also called the C3d receptor). This receptor is normally involved in B-cell activation by antigen, antibody and complement complexes. Activated B-blasts secrete cytokines and lymphokines that can stimulate B cell proliferation and express the corresponding receptors (e.g., CD 23), creating an autocrine loop. Moreover, the virus encodes three membrane proteins, LMP1, 2a and 2b, of which at least two control and modulate incoming signals, that participate in Ig-receptor activation, TNF-response and programmed cell death. As discussed in more detail below, the LMPs are constitutively active multifunctional membrane proteins. LMP1 interferes with TNF-α signaling. It can replace many CD40 induced functions and activates major signaling systems in B-lymphocytes and epithelial cells, such as NFkB, JNK-kinase and one JAK/STAT-pathway. Protection from apoptosis is one of its major downstream effects. LMP2a modulates kinase signaling from membrane receptors. Most notable are the eight N-terminal phosphotyrosine motifs that interact with the Ig-receptor induced kinases lyn and syk. LMP2a has an Immunoglobulin Transactivation Motif (ITAM) – with complete homology to the corresponding Ig-receptor ITAM-motif of its gamma-chain, that binds the syk kinase in its activated, phosphorylated state. Interspersed with these motifs are a PPPPY-motif that interacts with WW-domains of the Nedd-family of E3-ubiquitine ligases (Winberg *et al.*, 2000). It is conceivable that LMP2a may attract the kinases and is involved in their fast destruction by guiding the complex to the ubiquitine –proteasome system. Characteristically, the expression of

Table 29.2. Overview of the EBNA proteins

Name	Sub-type	Viral functions	Interactive cellular proteins	Expression	Required for in vitro transformation
EBNA-1		Maintains viral episomes Regulates viral promoters	Karyopherins 2 α and β; TAP/p32; USP7 (HAUSP); RPA	All EBV-carrying cells	Yes
EBNA-2	A& B	Activates viral and cellular promoters	PU.1; hSNF5; Spi-B; RBP-J kappa; p300/CBP; DP103; p100; TFIIE;TFIIH; TFIIB; TAF40; myb; TBP	Latency III in B-cells	Yes
EBNA-3 (EBNA-3A)	A& B	Represses the RBP-J kappa dependent transcription	RBP-J kappa; RBP-2N; CtBP; epsilon-subunit of TCP-1; XAP-2; F538 (UK/UPRT); AhR	Latency III in B-cells	Yes
EBNA-4 (EBNA-3B)	A& B		RBP-J kappa; RBP-2N	Latency III in B-cells	No
EBNA-5 (EBNA-LP)		Enhances EBNA-2 dependent transcription	Hsp27; Hsp70 (Hsp72); Hsc70 (Hsp73); HAX-1; HA95; alpha & beta tubulins; prolyl-4-hydroxylase alpha-1 subunit; p14ARF; Fte-1/S3a	Latency III in B-cells	Yes
EBNA-6 (EBNA-3C)	A& B	Represses the RBP-J kappa dependent transcription	RBP-J kappa; RBP-2N; DP103; ProT-alpha; SMN; NM23-H1	Latency III in B-cells	Yes

these proteins still permits the corresponding physiological signaling pathways to operate.

For example, LMP1 can replace CD40 ligand-CD 40 signalling, but does not interfere with physiological CD40-reception. LMP2 does not completely abolish Ig-receptor signaling, although it is likely to increase the signal threshold. It may be noted that the HHV8 membrane proteins K 1 and K 15, in combination, carry many of the motifs that function in the LMPs, such as the NFkB activation site and ITAM-motifs. Their role in latency, lytic cycle and tumorigenesis remains to be elucidated.

The following section will deal with the EBV-encoded growth transformation associated proteins in some more detail.

Growth transformation associated EBV encoded proteins

Table 29.2 summarizes the information of the six nuclear proteins (EBNAs).

EBNA1 is encoded by the ORF/KBRF1. It is a DNA binding protein of highly variable size (60–100 kD) due to the presence of a glycine alanine repetitive sequence, inserted in the first molecule that is flanked by a highly basic domain. The C-terminal part of the protein contains a stretch of acidic amino acids. It is expressed in most EBV-carrying cells, with the possible exception of latently infected resting B-cells (Reedman & Klein, 1974; Lindahl *et al.*, 1974; Andersson-Anvret *et al.*, 1978; Hennessy & Kieff, 1983; Dillner *et al.*, 1986a,b; Chen *et al.*, 1995a,b). In all other cell types that have been studied, EBNA 1 is expressed irrespectively of the cell phenotype, level of differentiation or, in the case of lymphocytes, activation status (Niedobitek *et al.*, 1989; Hamilton-Dutoit *et al.*, 1991; Prevot *et al.*, 1992; Zhou *et al.*, 1994). It is the only latency associated EBV-encoded protein whose expression is not influenced by the cell phenotype. In somatic cell hybrids between EBV carrying immunoblasts that express the full type III program of six nuclear and three membrane proteins and EBV negative non-B cells where all B-cell specific markers are eclipsed, EBNA1 but not EBNA 2–6 remains expressed (Contreras-Salazar *et al.*, 1989). EBNA1 is also the only member of the EBNA-family that remains associated with the chromosomes in metaphase (Ohno *et al.*, 1977; Jiang *et al.*, 1991). It is randomly distributed among the chromosomes, but binds specifically to the origin of latent viral DNA replication (*OriP*). This binding is necessary for the maintenance of the EBV episomes, by equal distribution to the daughter cells in mitosis (Jones *et al.*, 1989). EBNA 1 has three specific binding regions in the viral DNA, each multiple. 20 binding sites are in the family of repeats (FR), four in the dyad symmetry, and two downstream of the Q promoter (Reisman & Sugden, 1986; Sugden & Warren, 1989; Ambinder *et al.*, 1990; Rawlins *et al.*, 1985).

The latent replication of the viral DNA starts from ori P. EBNA1 binds to ori P as a dimer. It is composed of a flanking domain and a core domain. The flanking domain

includes a helix that projects into the major DNA grove and an extended chain that travels along the minor grove. This motif is responsible for all sequence determined contacts with DNA. The core domain makes no direct contact with DNA (Polvino-Bodnar *et al.*, 1988). The binding to chromatin is mediated via chromatin protein (ref). EBNA1 binding to the chromosomes is essential for the precise division of the replicated DNA into the two daughter cells.

Through the multiple interactions with viral DNA, EBNA 1 causes DNA looping by multimerization. This increases the complexity of its promoter regulation. Dyad symmetry controls S-phase associated viral DNA replication. EBNA 1 regulates viral promoters via its multiple binding sites. FR acts as an enhancer for the C-promoter, directing all six EBNA transcripts and the Qp elements that are negative regulators of Qp-driven EBNA1 transcription through a negative feedback loop (Bodescot *et al.*, 1987; Sample & Kieff, 1990).

EBNA1 contains a glycine–alanine repeat of variable length that inhibits its processing through the proetasomes and the subsequent MHC class 1 association of the derived peptides, a prerequisite for recognition by CD8 positive cytotoxic T-cells (Levitskaya *et al.*, 1995). This results in a dramatically extended half life of EBNA 1 to more than 2 weeks, and may contribute to its presence in resting B-cells without *de novo* synthesis.

EBNA2

EBNA2 (ORF:BYRF1) is an 82 kD phosphoprotein. It contains a 14 AA long domain that is responsible for transactivation. In contrast to EBNA1, the expression of EBNA2 is restricted to immunoblasts (Dillner *et al.*, 1985; Hennessy & Kieff, 1985; Ernberg *et al.*, 1986). On primary infection of B-cells it acts as a transcriptional transactivator (Rickinson *et al.*, 1987). It is essential for the transformation of B-cells into immunoblasts and the derivation of LCLs (Cohen *et al.*, 1989). EBNA2 defective viral substrains cannot activate B-cells. EBNA2 is the EBV encoded oncoprotein that differs most extensively between EBV types 1 and 2 (Zimber *et al.*, 1986). Type I EBV is a more efficient transformer of primary B lymphocytes than type 2 (Rowe *et al.*, 1989). EBNA2 is associated with nucleoplasmic, chromatin and nuclear matrix fractions.

EBNA2 induces a variety of activation markers and other cellular proteins in B-cells, including CD23, CD21, c- fgr and c-myc. It is required for the expression of EBV encoded LMP1 and LMP2a in immunoblastic cells (Wang *et al.*, 1987a,b, 1990; Aman *et al.*, 1990).

The interaction of EBNA 2 with the cellular proteins p300 and CBP is critical for EBNA2 mediated transactivation, due to the intrinsic histone acetylase activities of the former and their interaction with transcription factors (Bornkamm & Hammerschmidt, 2001). CBP has been implicated in EBNA2 activation of c-myc promoter.

Even though EBNA2 is a potent activator of many cellular and viral genes, it does not bind directly to DNA. It influences the responding promoters through its interaction with RBP-Jk, PU1 and other cellular proteins. The complexes formed modify the affinity of histones for DNA. Further chromatin remodeling activity is achieved through an interaction between EBNA2 and hSFN5. Recruitment of EBNA2 to DNA is essential for the transforming activity of EBV and RBP-Jk is the most extensively studied partner. RBP-Jk functions as a downstream target of the cell surface receptor known as Notch. Notch genes encode cell surface receptors that regulate developmental processes in a wide variety of organisms. The cleaved product of Notch is targeted to the nucleus where it binds to RBP-Jk and can activate transcription, but with a lower efficiency than the intracellular part of Notch. The binding of ligand to the extracellular domain of Notch results in the cleavage of an intracellular domain. This intracellular fragment of Notch (Notch-IC) migrates to the nucleus, binds to DNA-bound RBP-Jk and converts thereby a repressor of transcription into an activator (Hsieh *et al.*, 1996).

On the basis of these findings, EBNA2 is regarded as a constitutively active homologue of Notch. However, Notch can only partially substitute for EBNA2 in B-cell transformation experiments, probably because it does not upregulate the transcription of LMP1 or c-myc. The EBNA2 induced activation of the LMP1 promoter requires additional B lymphocyte specific factors, such as PU.1 (Johannsen *et al.*, 1995) and RBPJK (Johannsen *et al.*, 1996). Elements responsible for EBNA2 responsiveness have been characterized in EBV-Cp, LMP1 and LMP2 promoters and the cellular promoter for CD23. All have at least one RBP-Jk binding site.

As already mentioned, the interaction of EBNA2 with the cellular sequence specific DNA binding protein, RBP-Jk, is critical for transformation and LCL outgrowth. A sequence in EBNA2 closely mimics a corresponding sequence in the notch-receptor. Notch and EBNA2 may activate transcription from the RBP-Jk and PU.1 promoters by interacting with SKIP, a component of the HDAC2 corepressor complex. The EBNA2 domain that interacts with PU.1 includes the site that interacts with RBP-Jk. The targeting of PU.1 by the EBNA2 transactivator is an important aspect of EBV adaptation to lymphoid cells (Tamura *et al.*, 1995).

The essential role of EBNA2 in the immortalization of B-cells is thus due to its transactivation of viral promoters

(Cp, LMP1 and 2) and a variety of cellular genes associated with B-cell activation and growth, among them c-myc. Myc activation in lymphocytes induces protein synthesis and increase in cell size, D-type cyclins, cyclin E. It downregulates the inhibitors p21 and p27. The induction of c-myc is regarded as a major link between EBNA2 and the cell cycle machinery (Kaiser *et al.*, 1999).

EBNA2 is also required to maintain the EBV driven proliferation of B-cells, as shown in the conditional LCL designated as EREB2–5, that contains an EBNA2-estrogen receptor fusion protein. The removal of estrogen from the growth medium results in cell cycle arrest and apoptosis. Early reintroduction of estrogen stimulates renewed cell cycle entry and proliferation (Kempkes *et al.*, 1995). EBNA2 can be replaced by the constitutive expression of exogenous c-myc. The switch from the EBNA2 driven to the myc-driven state is accompanied by a phenotypic change of the LCL-like cell to a more BL-like cell, resembling dividing germinal center B-cells (Polack *et al.*, 1996).

Paradoxically, EBNA2 can also inhibit proliferation in established BL lines. EBNA2 is a transcriptional repressor of the immunoglobulin mu-gene. In BL lines where myc is controlled by the immunoglobulin mu enhancer, EBNA2 expression results in suppression of the myc transcription from the translocated myc gene, leading to growth arrest (Jochner *et al.*, 1996).

EBNA5 (alternative name: **EBNA-LP)**. **EBNA5** is a nuclear phosphoprotein that localizes to distinct subnuclear bodies. It is spliced from a variable number of W1-W2 exon pairs, 66 and 132 nucleotides long, respectively, forming 66 aminoacid long repeats and, from the Y1 and Y2 exons, a C-terminal 45 AA unique region. Its progenitor, the giant primary transcript originates from the W or C promoter in immunoblastic cells (and only there) (Dillner *et al.*, 1986a,b; Wang *et al.*, 1987a,b; Bodescot & Perricaudet, 1986). Together with EBNA2, EBNA5 is the earliest viral protein expressed in freshly infected B-cells. The two proteins can induce the entry of resting B-cells into the G1 phase. Coexpression of EBNA5 with EBNA2 enhances EBNA2 mediated transcriptional activation. EBNA5 is tightly associated with the nuclear matrix, and often accumulates in PML bodies (Pokrovskaja *et al.*, 2001). It migrates together with various components of the proteasome dependent degradation machinery in heat shocked cells, and in cells treated with proteasome inhibitors, raising the possibility that EBNA5 participates in the regulation of specific protein degradation in the nucleus. Kashuba *et al.*'s experiments on EBNA5 also showed that it does not bind to Rb and p53 in the yeast two hybrid assay, but can exert an inhibitory effect on the p53-Rb axis by targeting the p53 regulator p14 ARF(Kashuba *et al.*, 2003). The latter can bind MDM2, suppress its ability to mediate in the degradation of p53 and thereby increase the expression level of p53. It was suggested that EBNA5 participates in the elimination of the p14 ARF-HDM2- p53 complexes and thereby contributes to the downregulation of p14 ARF and p53 protein levels in EBV infected B-cells (see also Kanamori *et al.*, 2004).

EBNA3 family

EBNA3 (ORF: BLRF3 + BERF1), EBNA4 (ORF: BERF2a + BERF2b) and EBNA6 (ORF: BERF3 + BERF4) are three large nuclear phosphoproteins in a size range of 140–180 kD. EBNA3 and EBNA6, but not EBNA4 is necessary for in vitro transformation. Individual sequences of these three EBNAs show little similarity, but they are all composed of a highly charged N-terminal half and a C-terminal half that contains numerous repeat elements. EBNA4 also contains an LxCxE motif. **EBNA6** is transactivator that induces CD21, 23 and LMP1 (alternative nomenclature: EBNA3a,b,c). All three proteins are encoded by tandemly arranged genes, localized in the middle of the viral genome (Hennessy *et al.*, 1985; Dillner *et al.*, 1986a,b; Shimizu *et al.*, 1988). They are all highly hydrophilic. They are stable proteins that accumulate in intranuclear clumps, sparing the nucleolus. They are believed to act as transcriptional regulators and can interact with RBP-Jk (Radkov *et al.*, 1997; Zhao *et al.*, 2003; Hickabottom *et al.*, 2002). The three proteins use unrelated peptide sequences for their interaction. EBNA3 and 6, but not EBNA4, are required for B-cell transformation (Parker *et al.*, 1996). By and large, the EBNA 3 family member, have similar but more limited effects on cellular gene expression compared to EBNA 2. EBNA4, as also EBNA3 and 6, generate highly immunogenic peptides that can associate with MHC class I molecules. Some peptide-HLA class I combinations can induce CD8+ CTL mediated rejection in immunocompetent hosts.

The following further information on the two members of the EBNA 3 family involved in growth transformation, EBNA 3 and 6, are of interest.

EBNA 3 (alt: EBNA 3a)

Kashuba *et al.* (2000, 2002) have identified two EBNA3 interacting proteins, using a two-hybrid technique. TCP-1 is part of a chaperonine complex (Kashuba *et al.*, 1999). EBNA3 binds to the epsilon subunit of TCP-1. Kashuba *et al.* proposed that nascent EBNA3 is folded by the TCP-1 containing chaperon complex through its binding to the apical region of the epsilon subunit. EBNA3 may thereby receive help for its proper folding.

Table 29.3. Overview of the EBV latent membrane proteins

Name	Variants	Functions	Major protein interactions	Expression	Necessary for in vitro transformation
LMP1	Latent form	Mimics CD 40 Activation of NFkB, JNK-kinase, JAK/ STAT, MAP kinase, Akt cell survival induction of adhesion and immune regulatory membrane proteins	TRAF 1, 2, 3 TRADD BRAM 1 LMP2A	B-cell latency II-III, BL, HL, DLBCL, T-cell lymphoma, NPC	Yes
	Lytic	Not known	Not known	Lytic cycle	No
LMP 2	LMP2A	Interacts with phosphotyrosine kinases incl Src-family and PI3-kinase Blocks lytic cycle Block BCR activation	Src, Lyn, Lck ZAP-70, Syk, AIP4/Nedd 4	B-cell latency I-III, HL, DLBCL, T-cell lymphoma, NPC	No
	LMP2B	Blockc LMP 2A?	Not known	B-cells latency III, DLBCL, NPC	No

Kashuba *et al.* (2000) also found that EBNA3 interacts with p38/XAP-2. In the presence of EBNA3, the cytoplasmic p38/XAP-2 translocates to the nucleus. XAP-2 also binds to the hepatitis B virus X antigen, believed to be involved in the oncogenic effect of hepatitis B virus. XAP-2 is known to be involved in the regulation of the aryl hydrocarbon receptor (AhR) pathway. AhR is a ligand activated transcription factor, a member of the HLH transcription family.

EBNA3 was also shown to interact with a new member of the UK/UPRT (uridine kinase/uridine phosphoribasyl transferase) family (Kashuba *et al.*, 1999). The predominantly cytoplasmic enzyme translocates to the nucleus in the presence of EBNA3. It was suggested that EBNA3 may influence the uridine salvage pathway by contributing to the increase of the nuclear UTP pool, required for active cell proliferation.

EBNA6 (alternative EBNA3c) is the only member of the EBNA3 family that has a leucine zipper (West *et al.*, 2004). EBNA6 associates with histone deacetylase and can repress transcription through the Notch signaling pathway. EBNA6 is unique among the EBNAs in its ability to coactivate the LMP1 promoter with EBNA2 (Lin *et al.*, 2002). EBNA6 has also a number of specific repressive effects (Touitou *et al.*, 2001; Radkov *et al.*, 1999). Moreover, EBNA6 associates with histone deacetylase and can repress transcription through the Notch signaling pathway (Radkov *et al.*, 1999).

EBNA6 can also cooperate with oncogenic mutant H-ras in the immortalization and transformation of REFs (Parker *et al.*, 2000). It can also override the suppression of this transformation by p16, by targeting the checkpoint at the G1/S transition, regulated by Rb. EBNA6 can induce aberrant nuclear division that results in multinucleated cells, polyploidy and eventually cell death (Allday review, sid.36). All this suggests that EBNA6 may disrupt multiple cell cycle checkpoints and produce a similar phenotype as the K cyclin of KSHV (Krauer *et al.*, 2004).

Table 29.3 summarizes some of the known interactions of the EBNAs with cellular proteins:

The latent membrane proteins (LMP) of EBV

In the course of infection, replication or persistence, viral gene products frequently interact with proteins that regulate signaling pathways in the host cell. This capacity to modify host cell signal transduction is particularly apparent in the control of EBV.

EBV can express three membrane proteins during latent infection, latent membrane protein (LMP) 1 and 2 A and B (LMP 2A and B) (Hennessy *et al.*, 1984; Laux *et al.*, 1988; Longnecker & Kieff, 1990). These proteins interfere with multiple cellular signal transduction pathways so as to modulate apoptosis and cell surface receptor signalling. They are both transmembrane proteins with six (LMP 1) or twelve (LMP 2) anchoring transmembrane domains, according to computer based structure predictions. No full crystal structures have been established. Neither one acts as a ligand-receptor, but through constitutive activation at cellular membranes (Gires *et al.*, 1997). If there are no ligands, why are they located as membrane proteins?

The main function of both membrane proteins appears to be directed towards interaction with signalling and adaptor molecules normally regulated by cell membrane receptors. They are highly multifunctional and interact with several cellular signalling pathways. Importantly both proteins are expressed at the cell surface membrane as well as in intracellular membranes of the Golgi and endoplasmic reticulum (Lynch *et al.*, 2002; Eliopoulos &

Young, 2001). The significance of this compartmentalization is not known, since the function of the intracellularly localized LMPs has not been the subject of focused studies. Both proteins can be expressed in two forms, one full length and one shorter variant where the first (LMP 1) or the last exon (LMP2B) is excluded by alternative promoter usage or alternative splicing. The shorter variants appear structurally competent to disrupt or block the constitutive activation of the full length protein, by interfering with activation mechanism. LMP 1 is activated by aggregation to trimeres or multimeres, mediated by the transmembrane domains. The truncated protein variant lacks the C-terminus and four of the transmembrane domains. LMP 2 depends on the N-terminal tail and its phosphorylation for activation, which cannot take place with LMP 2B. Both LMP1 and 2 are expressed in latency forms II and III immunoblasts and derived tumors and cell lines (Rea *et al.*, 1994). LMP2A transcripts are also expressed in resting virus carrying B-lymphocytes in healthy individuals, the reservoir of persistently latent EBV (Chen *et al.*, 1995a,b; Qu & Rowe, 1992; Tierney *et al.*, 1994). Both proteins are also detected in epithelial tumors of the nasopharynx (NPC) and during the early stages of oral hairy leukoplakia (Pathmanathan *et al.*, 1995; Webster-Cyriaque & Raab-Traub, 1998). In NPC between 35 and 65% of the tumors are LMP-positive. LMP1 and 2 are expressed in a coordinate fashion Their transcription is co-regulated via a 600 bp bi-directional promoter-enhancer control element designated as the LMP-regulatory sequence (LRS). LMP 2 can only be expressed from viral episomes, since the precursor transcript passes through the terminal repeats (Table 29.3).

The short variant of LMP1 has only been demonstrated during productive, lytic virus infection where it may block the constitutive action of full length LMP 1.

Latent membrane proteins 1 (LMP1)

LMP 1 is a 356 amino acid protein with a short intracellular N-terminus and 150 aa C-terminus.

LMP1 is essential for the transformation of B lymphocytes into lymphoblastoid cell lines (Dirmeier *et al.*, 2003). It confers a survival advantage on EBV-infected B cells by protecting them from apoptosis. This is largely due to the LMP1-induced upregulation of the anti-apoptotic protein Bcl-2 and the block of p53 mediated apoptosis by the latter (Henderson *et al.*, 1991).

EBV-encoded LMP1 can also transform established rodent fibroblasts in vitro (Baichwal *et al.*, 1989; Fahraeus *et al.*, 1990). Furthermore, expression of LMP1 is correlated with a more favorable influence of treatment (chemotherapy/irradiation) on patients with NPC (Kawanishi, 2000) or Hodgkin's lymphoma (Montalban *et al.*, 2000). Clinical and follow-up data from 74 cases of NPC showed that LMP1 positive NPC grew faster and more expansively than LMP1 negative tumors.

LMP 1 almost completely mimicks the function of CD 40 mediated signalling and is thus functionally homologous to the TNF receptor (TNFR) family of proteins (Eliopoulos *et al.*, 1996, 1997; Kilger *et al.*, 1998; Zimber-Strobl *et al.*, 1996; Lam & Sugden, 2003). LMP1 has been shown to interact with several proteins of the TNFR signaling pathway through its C-terminal activation region (CTAR) 1 and 2. Hence, LMP1 can bind TRAF (TNFR-associated factor) 1, 2 and 3, as well as TRADD (TNFR-associated death domain protein), an adaptor protein that serves to recruit caspases to the death-inducing signaling complex (DISC) of the TNFR (Mosialos *et al.*, 1995; Devergne *et al.*, 1996). These interactions result in the NFκB-dependent upregulation of a number of genes, including those encoding anti-apoptotic proteins such as A20 and Bcl-2 (Hatzivassiliou, 2002). Bone morphogenetic protein receptor IA-binding protein (BRAM1), a novel LMP1-interacting protein, interferes with LMP1-mediated NFκB activation and reverses the resistance of cells to TNFR-mediated apoptosis (Chung *et al.*, 2002). Kawanishi (2000) has provided evidence that LMP1 domains CTAR1 and 2 are involved in the enhancement of TNF-induced apoptosis in epithelial cells.

It has been shown that EBV also modulates host apoptotic sensitivity by modifying the relative level of caspase-8, an initiator caspase and its competitor, FLIP (FLICE inhibitor protein (Tepper & Seldin, 1999). LMP1 may alter the ratio of caspase-8 and FLIP.

The findings of Zhang *et al.* (2002) suggest that the apoptotic modulation by LMP1 is stimulus dependent: tumor necrosis factor (TNF) induced apoptosis was inhibited while Fas ligation- and etoposide- induced apoptosis was potentiated. The attenuation of TNF induced apoptosis partallelled the induction of the anti-apoptotic zinc finger protein A20.

LMP 1 also induces IL 6 and IL 6-receptor expression via the JAK/STAT pathway and JNK-kinase and MAP-kinase (Eliopoulos *et al.*, 1997; Gires *et al.*, 1999; Kieser *et al.*, 1997). Through its interference with a number of major signaling pathways in B-cells and epithelial cells, LMP 1 mediates deregulation of several hundred cellular proteins. LMP 1 also induces the expression of adhesion molecules such as ICAM-1 and LFA, and MHC Class I and II (Mehl *et al.*, 2001; Rowe *et al.*, 1995).

LMP2 modulation of signaling in B-cells and epithelial cells

LMP2A has an intracellular N-terminal cytoplasmic region of 119 residues, which is predicted to be followed by 12 membrane-spanning regions and a short C-terminal cytoplasmic tail, also intracellular. It has been reported to aggregate into "cap-like" structures at the plasma membrane and specifically associate with lipid rafts (Dykstra *et al.*, 2001; Higuchi *et al.*, 2001). The C-terminal tail of LMP2A has been reported to possess a clustering signal as well (Matskova *et al.*, 2001).

LMP2, along with EBNA1 and LMP1, is consistently detected in some latently infected B cells and EBV-associated diseases in vivo, and plays presumably important roles in vivo, related to viral replication, persistence and EBV associated diseases (Qu & Rowe, 1992; Miyashita *et al.*, 1997). A major role of LMP2A in relation to latent EBV infection may stem from its ability to inhibit the activation of lytic EBV replication by cell-surface-mediated signal transduction (Miller *et al.*, 1994). This may prevent lytic replication in latently infected B-cells as they circulate in the blood, bone marrow or lymphatic tissues, where they might encounter antigens, superantigens or other ligands capable of engaging B-cell receptors and activating the viral cycle. In this context it may be relevant that LMP 2A also downregulates telomerase birth in B-cells and epithelial cells (Chen *et al.*, 2004; Scholle *et al.*, 2000).

LMP2A's ability to interfere with BCR signaling and to maintain viral latency may stem from protein–protein interaction motifs located within the amino-terminal tail (see Fig. 29.2). These include a YEEA (amino acid; single letter code) site that, when phosphorylated on the tyrosine residue (Y112), can serve as a binding site for the Src Homology 2 (SH2) domain of the Src family tyrosine kinase Lyn (Burkhardt *et al.*, 1992; Miller *et al.*, 1994; Frueling *et al.*, 1998). In addition, LMP2A possesses an immunoreceptor tyrosine-based activation motif (ITAM) with the consensus sequence YXXI/V-$X_{(6–8)}$-YXXI/V which is found in a number of immunoreceptors including the BCR, the T-cell receptor (TCR), as well as the Fcε receptor that binds IgE. This motif in LMP2A, when phosphorylated on tyrosines 74 and 85, provides a binding site for the dual SH2 domains of the tyrosine kinase Syk (Fruehling & Longnecker, 1997). LMP2A also possesses 2 PPPPY (PY) motifs that can bind to the WW domains of the NEDD4 family of E3 ubiquitin ligases including AIP4, NEDD4-2, and WWP2 (Winberg *et al.*, 2000; Ikeda *et al.*, 2000). Binding to NEDD4 proteins is abrogated by mutation of both tyrosines in the PY motifs (Y60; Y101). NEDD4 family proteins contain HECT (homologous to E6-associated protein carboxy-terminus) domains that catalyze the ubiquitination of proteins such as those associated with the WW domains and target them for degradation via either the 26S proteasome or a lysosomal pathway. LMP2A and the LMP2A-associated kinases are substrates for the NEDD4 family of proteins, suggesting that LMP2A may not only sequester tyrosine kinases away from the BCR, but may also direct them to ubiquitin-mediated pathways including degradation.

Somewhat paradoxically, LMP2A has also been shown to mimic BCR signaling. When expressed as a B-lineage specific transgene in mice, it can both drive B-cell development and promote the survival of mature B cells in the absence of surface immunoglobulin (Ig) expression (Caldwell *et al.*, 2000). Furthermore, this signal appears to be attenuated by the NEDD4 family protein, Itch, indicated by the finding that Itch −/− introduced into the LMP2A transgenic background enhanced LMP2A-mediated signaling (Ikeda *et al.*, 2003). This suggests that LMP2A may act as a survival factor for EBV-positive B-cell tumors that have lost the expression of surface Ig, while also preventing virus reactivation, such as in HL.

LMP2A has also been shown to activate PI3 kinase and the downstream phosphorylation of Akt in epithelial cells and B cells. This may result influence cell growth and apoptosis (Swart *et al.*, 2000).

EBV LMP2A and HHV8 K1 and K15 membrane proteins both target the BCR, but in different ways. While the LMP2A effects on cellular signal transduction have been widely studied, the functions of the HHV8 membrane proteins have been given equal attention (Choi *et al.*, 2000; Lee *et al.*, 2000).

The transforming HHV8 K1 transmembrane transforming protein associates spontaneously to form a trimer in the membrane, which, like LMP2A, carries ITAM motifs. K1 prevents cell surface expression of the BCR by the association of its N-terminal domain with the μ-chains of the BCR, thus preventing the CD79α and β chains from binding. The K1-BCR complex is retained in the ER. The ITAM-motif of K1 is thus available for interaction with cytoplasmic signaling proteins. It has been speculated that the ITAM motif is speculated to function by delivering growth and survival signals to the target cell (Lee *et al.*, 2000). The K15 has a similar topology as LMP2A, with 12 transmembrane helices, but lacks the N-terminal signal transduction domain of LMP2A. It reportedly blocks BCR signaling but the mechanism is not known.

Thus, similar functional elements of function appear to have been conserved in the latent membrane protein of these two distantly related herpesviruses, although the protein structure differs.

In view of the puzzling fact that K1 appears to bind to the μ-chain of BCR in the ER, with intracellular retention of the complex as a result, it is important to determine whether its ITAM motifs bind the Syk tyrosine kinase or a different SH2-domain protein. This could answer the question how K15 can stimulate B-cell survival and proliferation.

The elucidation of the function of the gamma herpesvirus signal transduction mediators may thus allow a refined understanding of BCR signaling functions, by way of a perturbation analysis.

Immunoblastic lymphomas arise in bone marrow or organ transplant recipients (PTLD), congenital immunodeficiencies, particularly the X-linked lymphoproliferative syndrome (XLP) and in AIDS patients. In PTLD and XLP, EBV carrying immunoblasts proliferate, as in mononucleosis, but without being arrested by the immune response. Initially, the proliferation may be polyclonal but becomes eventually monoclonal. During the polyclonal phase, the progression of the disease can often be halted by viral DNA inhibitors such as acyclovir, indicating that virus release and recruitment of new virally transformed cells play a role in the initial development of the disease, but not after it has turned into a monoclonal lymphoma.

EBV carrying immunoblastomas express the full (type III) set of the virally encoded growth transformation associated antigens. They provide the virus carrying B-cells with proliferation drive and antiapoptotic protection. They include the highly immunogenic members of the EBNA3 triad (EBNA 3,4 and 6, also called EBNA 3a,b,c), explaining why passive immunotherapy with sensitized CD8+CTLs or with unsensitized but immunocompetent, histocompatible T-cells can bring about dramatic regression even of widely disseminated tumors.

The immunoblastomas and lymphomas that arise in AIDS patients show a broader picture. Only part of them resemble the post-transplant immunoblastomas. Others are more akin to EBV carrying Burkitt lymphomas, mainly because they carry BL-type Ig/myc translocations, as discussed in more detail below, but they have a more variable cellular and viral expression phenotype. In the strict (type I) phenotype, associated with high endemic BL (only EBNA1 and the EBERs are expressed), Ig/myc translocation carrying tumors with a more immunoblastic cellular and viral (typeIII) expression phenotype are also found. This may be related to the well established fact that EBV-carrying type I BL cells tend to drift towards a more immunoblastic phenotype and full (type III) antigen expression during in vitro culturing. The selective filter that normally removes the highly immunogenic immunoblasts is, not surprisingly, impaired in immunodefectives.

EBV and Burkitt lymphoma (BL)

EBV is associated with 98% of the high endemic BLs, but only with about 20% of sporadic BLs. Both types contain essentially similar Ig/myc translocations, with only minor differences (Magrath, 1990). Only EBNA1 and the EBERs but none of the growth transformation associated EBV proteins (EBNA 2–6, LMP1–2) are expressed in BLs. The virus can therefore not be held directly responsible for the proliferation of the tumor. The latter function is attributed to the constitutive activation of the c-myc protooncogene, resulting from its juxtaposition to one of the three Ig-loci by chromosomal translocation.

Phenotypically, BL cells differ from EBV transformed or mitogen activated immunoblasts. Their markers and their V-gene mutations identify them as post–germinal center memory cells. Even in the most highly BL prone areas of Africa where malaria is rampant and where the regularly high EBV antibody levels in most young children indicate early infection and a high viral load – proven risk factors for the development of BL (Geser *et al.*, 1982) – only a very small fraction of normal B cells (<0.1%) carry the virus. The 98% EBV positivity of the BLs must therefore mean that the presence of the virus increases the probability of lymphoma development. This is the same as to say that the virus contributes to the development of the tumor.

Falling short of the activation of cell proliferation, EBV is likely to contribute to the genesis of BL in some other way. According to one theory, apoptosis protection by the EBERs may be responsible (Takada, 2001). Another indication of a possible apoptosis protecting role has been derived from experiments with EBV negative sublines of originally EBV positive BLs that have lost the virus accidentally, or after hydroxyurea treatment. Viral loss is accompanied by decreased clonability and tumorigenicity (Komano *et al.*, 1998). Comparison of three independently established EBV carrying BLs and their EBV–loss variants showed a marked downregulation of the tcl-1 oncogene (Kiss *et al.*, 2003). Tcl-1 is highly expressed on both T and B cell derived leukemias. EBV reinfection has upregulated tcl-1 again in the EBV-loss variants. Since originally EBV negative BLs express tcl-1 at a high level, we have suggested that the EBV negative BLs switch on tcl-1 constitutively during their neoplastic development. In the virus carriers, EBV is responsible for the upregulation. Tcl-1 activates the apoptosis protective AKT pathway and may thus further increase the apoptotic threshold in these myc-driven and thereby apoptosis prone cells. Conceivably, EBV may also act at the pretranslocation level, by contributing a strong B-cell proliferation, driving stimulus. This would be further enhanced by malaria associated immune dysregulation

and opportunistic coinfections. This could expand the target population available for the critical myc-translocation.

The absence of the virus from the EBV negative BLs is consistent with the interpretation that the virus is a contributory, but not a mandatory factor for BL development. The Ig/myc translocation is, on the other hand, regularly found in all BLs and must therefore play a more central role in the origin of the tumor.

The Ig/myc translocation carrying murine tumor prototype, plasmacytoma (MPC) develops earlier and in a higher frequency when the precursors are infected with the pre-B-cell-immortalizing Abelson virus, that carries the v-abl oncogene that has a known antiapoptotic effect.

Since myc-driven cells are highly apoptosis prone, as already mentioned, it is hardly surprising that BL cells are protected against apoptosis at several different levels. Both the Rb and p53 pathways are crippled in BLs, as a rule. In most cases, the Rb pathway is impaired by p16 promoter hypermethylation. The p53 pathway can be inactivated in at least three alternative ways: p53 mutation, ARF mutation or deletion, and MDM2 amplification (Lindström & Wiman, 2002). In spite of this. the BL cell is still quite apoptosis prone, as indicated by its "starry sky" histology, where the "stars" are macrophages that have engulfed apoptotically generated nuclear fragments from the lymphoma cells, and also by the high chemotherapeutic sensitivity of the tumor.

The primary localization of African Burkitt lymphomas may be relevant in this context as well. It suggests that the cytokine environment associated with local tissue proliferation may favor the outgrowth of Ig/myc translocation carrying cells. Jaw tumors are frequent around the age of dentition, ovary and testis are frequent primary sites of BL in prepubertal and pubertal children. Lymphomas may arise in the long bones of teenagers, and BLs with a primary mammary localization have been seen in young lactating women. Some of them regressed when nursing was interrupted. Chronic inflammation may act in a similar way. EBV carrying body cavity lymphomas can be associated with pyothorax of 20 or more years' duration. They carry HHV-8 as well. They present as diffuse large cell lymphomas, sometimes with an immunoblastic appearance. This is in line with numerous examples in the experimental literature, showing that chronic inflammation may act as a tumor promotor.

Hodgkin's lymphoma (HL)

Almost half of the HL-cases in Western countries carry EBV-positive Hodgkin Reed-Sternberg (HRS) cells, that express EBERs, EBNA 1, LMP1 and presumably LMP2a, although this has been less extensively studied (Glaser *et al.*, 1997; Levine *et al.*, 1994; Ohshima *et al.*, 1996; Lennette *et al.*, 1995). The frequency of these presumably malignant cells is surprisingly low in the tumors (1–3%). Conceivably, the HRS cell orchestrates tissue derangement, by recruiting immune bystander cells such as non-neoplastic helper T lymphocytes, plasma cells, macrophages and eosinophilic granulocytes (Molin *et al.*, 2001; Enblad *et al.*, 1993; Weng *et al.*, 2003). It is frequently surrounded by a rosette of CD4+ T-lymphocytes of both the Th1 and the Th2 type. EBV positive HLs show a shift towards Th1. The tumor is described as a "malignant inflammatory process." It produces a large variety of cytokines (Dukers *et al.*, 2000). The HRS cells carry non-functionally rearranged immunoglobulin heavy chain genes and contain somatic mutations in a high frequency, indicating post-germinal center derivation (Kuppers *et al.*, 2002; Muschen *et al.*, 2000; Spieker *et al.*, 2000). They may have been frozen in a non-physiological state that prevents further differentiation or apoptosis.

Several findings suggest that EBV positive HRS originate from latently EBV infected B cells. Like BL and NPC cells, HL cells carry complete viral genomes in the form of multiple covalently closed episomal DNA. TR analysis revealed that viral genomes were clonal, suggesting that they have originated in a common proliferating precursor (Langerak *et al.*, 2002). This argues against any role of virus replication in the establishment of the tumor cell.

Enhanced permissiveness and virus replication may still have a role as a risk factor, HD patients frequently have elevated antibody titres to EBV early antigen (EA) already at the presentation of disease and often years before. Significantly increased EBV genome load has been detected in the blood several years before the disease (Drouet *et al.*, 1999). This suggests that the disease may be preceded by increased EBV reactivation and deregulation of the virus-host balance. Moreover,it has been shown that patients with acute infectious mononucleosis run an increased risk of developing HD (Amini *et al.*, 2000; Axdorph *et al.*, 1999).

In contrast to BL-cells and LCLs, it is difficult to grow HRS-cells in vitro. Only a dozen HL-/HRS-dervied cell lines have been established in vitro. They are EBV-negative, with only one exception. EBV-positive and negative HLs show no convincing differences in phenotype or clinical behavior. But while HD is less common in developing countries, it is much more frequently EBV-positive, (up to 90%). This is reminiscent of the difference between African and sporadic BLs (98% vs. 20% EBV positives).

Two studies have shown no difference in the prognosis of EBV positive and negative cases of HD. Morente *et al.* (1997) found however, that EBV-positivity was associated

with improved overall survival and resulted in a higher complete therapeutic response, together with a significantly longer disease free interval. According to a fourth study (Murray *et al.*, 1999) EBV positive tumors are easier to treat and treatment leads to longer disease free intervals.

EBV and nasopharyngeal carcinoma

Low differentiated or anaplastic NPC is the most regularly EBV associated tumor. It is the commonest malignant tumor among men in Southern China, the Guangzhou region in particular. The carcinoma cells carry multiple viral episomes, like BL cells. Terminal repeat (TR) analysis revealed that they have been derived from a single infectious event like the EBV positive BLs.

Some apparent paradoxes need to be reconciled. Given that the virus replicates lytically in epithelial cells, as particularly well shown in oral hairy leukoplakia (OHL, see below), why does it remain latent in NPC where it expresses only EBNA1, the EBERs and the LMPs (latency II)? Is this due to the fact that NPC cells do not proceed to squamous differentiation? In OHL, the productive viral cycle is only switched on when the cells move upwards within the epithelium, to the level where they start engaging in squamous differentiation.

Early reports claiming that latent EBV could be detected in the basal layer of normal epithelia by in situ techniques have not been confirmed. Foci of lytic viral replication are only found in OHL, which is an EBV-induced lesion. It is found in immunodefectives and can be cursed by acylovir.

The robustly latent interaction between EBV and NPC is puzzling. It raises the question whether EBV inhibits the differentiation of NPC cells and, if so, whether this gives a clue to its role in the genesis of NPC.

It is not clear how and when the NPC-precursor cell becomes infected. In contrast to the EBV-infectability (and transformability) of B cells with EBV and the persistence of the virus in this compartment, epithelial cells are difficult to infect, unless a genetically engineered virus is used that carries a selectable marker like neomycin resistance.

Two experimental findings may offer possible clues. Comparing three different lines of EBV negative carcinoma cells with their EBV infected sublines, Nishikawa *et al.* (2003) found that the virus switched on the expression of a truncated basic hair keratin gene in all three lines. Conceivably, the truncated keratin may interfere with the production of full sized keratin and, thereby, differentiation.

But why would the keratin be truncated in the first place? This could stem from some of the multiple genetic changes found by PCR in NPC precursor lesions. Dolly Huang's group has shown that some of the changes, particularly the frequent deletions affecting chr.3p and 9p, occur prior to EBV infection (Lo & Huang, 2002). A possible scenario proceeds from an early genetic change that endows the precursor cell with the ability to produce a variant keratin, potentially capable of interfering with differentiation. This variant would be switched on by some viral product, expressed within the latency II program. From the viewpoint of viral strategy, such a scenario would protect the virus from self-elimination by lytic infection and secure its persistence in latently infected, dividing, and undifferentiated cells.

Viral expression in carcinoma cells, cell behavior and host relationships in NPC

About two thirds of NPC tumors express LMP1 in vivo. In the non-expressors, the promoter region of the gene is hypermethylated (Hu *et al.*, 1991). A comparison between non expressed LMP1 genes taken from NPC biopsies and the corresponding genes from LMP1 expressing tumors, showed that the former but not the latter could confer immunogenicity (rejection inducing capacity) on a non-immunogenic mouse mammary carcinoma, transplanted to syngeneic hosts (Hu *et al.*, 2000). This suggested that the LMP1 expressors may have been sculpted by immunoselection in vivo that favored cells with genetic or epigenetic LMP1 inactivation.

LMP1 has been shown to convey increased agarose clonability and tumorigenicity on immortalized epithelial cells in vitro (Hu *et al.*, 1993). Moreover, LMP1 expressing NPCs grew more expansively in immunodefective mice than non-expressors (Hu *et al.*, 1995). Nevertheless, patients with LMP1 positive tumors showed better survival in a retrospective study than patients with LMP1 negative tumors, suggesting that the immunoselective sculpting of the LMP1 positives may still have left the tumors with a certain residual immunogenicity.

In addition to NPC, EBV genomes were also found in other solid tumors, notably gastric carcinomas, salivary gland tumors and a case of thymic carcinoma. Since these associations are not equally regular, they will not be included in this comparative discussion.

Immune surveillance and the oncogenic herpesviruses – the role of immunological "anticipation"

Viewed as a group, four of the potentially oncogenic herpesviruses, Marek's disease herpesvirus (MDHV), *H.*

saimiri (HVS), *H. ateles* (HVA) and EBV provide us with some important lessons about surveillance mechanisms that can protect against viral tumorigenesis, without reducing the spread of the virus. This results in a largely non-pathogenic "live and let live" situation.

MDHV is the only known DNA tumor virus that can cause tumors by epizootic infection. Unlike the three other viruses, MDHV is not ubiquitous in its natural host, the chicken. Infection of immunologically naive birds can therefore cause horizontally spreading epizootics. The frequency of the lymphoproliferative tumors and the level of their malignancy is influenced both by genetic and evironmental (e.g., stress) factors. One of the genetic resistance factors is linked to MHC. This is in line with the known role of the locus as an immune response regulator. It is reasonable to assume that a more general spread of the agent in chickens would have selected the natural host species for a similarly solid resistance that has been achieved by the other three viruses.

HVS infection is ubiquitous in the natural New World primate host, the squirrel monkey, and the same applies for HVA and its natural host, the spider monkey. Neither of the two viruses are known to be associated with any disease. But the number of examined animals and the observation periods are not comparable to what is known about EBV and the human host. The effect of immune defects is not known for the two simian viruses either.

In distantly related but HVS and HVA naive New World primates, such as marmosets, both HVS and HVA cause rapidly proliferating T-cell malignancy. The tumorigenic potential of both viruses is also consistent with their ability to immortalize human T-lymphocytes.

What makes the natural host solidly resistant to the tumorigenic effect of transforming viruses that can cause rapidly growing malignancies in related but immunologically naive species? Some years ago we performed an experiment with Friedrich Deinhardt's group (Klein *et al.*, 1973) that may throw a certain light on this question. We compared the primary antibody response of squirrel monkeys and marmosets to HVS inoculation soon after birth. Since all previously tested squirrel monkeys were found to carry HVS, fully mature fetuses were removed by Cesarian section from their mothers and were kept virus free by isolation, surrogate mothers and bottle feeding. After a few weeks they were inoculated with live HVS, in parallel with young marmosets. Both species responded with antiviral antibody production but while the squirrel monkeys reached peak antibody titers already 10–12 days after inoculation, the marmosets started responding only after 3 weeks. By that time the lymphoproliferative disease was already in progress, however.

Even though this study was restricted to humoral antibodies and gave no information about the more important cell mediated arm of the immune response, the time difference in the onset of the reaction indicated that the natural host of the virus has been selected for some kind of "immunological anticipation" of the impact of HSV that succeeds in infecting most and perhaps all members of the species under natural conditions.

EBV is also ubiquitous in its natural human host and causes only a self-limiting proliferative disease, mononucleosis, and only in about half of the primarily infected adolescents and adults. The other half and most children under the age of ten respond with "silent," symptom-free seroconversion. Mononucleosis is characterized by the temporary proliferation of EBV transformed immunoblasts, followed by a rejection reaction, mediated by multiple immune effectors. In immunodefectives, whether of congenital (e.g., XLP) or iatrogenic (e.g., transplant recipient) or infectious (e.g., HIV) origin, immunoblastic proliferation may be fatal, as already discussed in the section on immunoblastomas. Moreover, and in further similarity to HVS and HVA, EBV can induce progressive lymphoproliferative malignancy in marmosets and owl monkeys, New World primates that do not have EBV-like viruses of their own. Old World primates that have their own EBV-like viruses carry cross reactive antibodies and are solidly resistant against EBV infection.

Neither BL nor NPC, nor any of the other malignancies where EBV is only found in a proportion of cases, are directly caused by EBV. Therefore, the relatively non-tumorigenic association of this ubiquitous virus with its natural human host is not very different from what HVS and HVA have achieved in their natural simian hosts. Our "immunological anticipation" of EBV is reflected by the surprisingly high frequencies of CD8+T-cells specific for latent and lytic EBV antigens in healthy virus carriers. As many as 5.5% circulating CD8+T cells in a virus carrier can be specific for a single EBV lytic protein epitope, as shown by tetramer staining (Tan *et al.*, 1999). Lymphocytes carrying TCRs specific for latent proteins were found at somewhat lower, but still considerable (0.4–3.8%) frequencies. The preparedness of the immune system for the encounter with EBV transformed B-cells is also reflected by the fact that autologous T cells, admixed to EBV infected B-cells, undergo blast transformation and generate cytotoxicity at equally high levels as allogeneic, MHC-incompatible mixed lymphocyte cultures.

Surveillance of the human host against the development of EBV carrying neoplasia is thus virtually watertight. Occasional tumor development is due to an immunological or cytogenetic accident. Directly EBV-driven immunoblastomas can only arise in immunodefective hosts. Burkitt

lymphoma is driven by the unrelated accident of the Ig/myc translocation, probably assisted by an anti-apoptotic effect of the minimal, non proliferation driving type I viral expression program. In NPC, the role of EBV is even less clear. Our findings prompt the speculation that it may act by inhibiting squamous differentiation.

HHV-8 (KSHV)

This is the most puzzling virus of the DNA tumor virus family. It has hijacked more than a dozen cellular genes, many of which have potential tumor related functions. They include genes that influence the cell cycle, apoptosis, various other types of signaling and can create chromosomal imbalance. If a tumor biologist had been given the task of designing a tumor virus, he could not have done better. Surprisingly, however, the virus does not transform in vitro any target cell so far tested. The lack of an in vitro transformation system has hampered the functional analysis of tumorigenicity. Still, there are very good grounds to believe that the virus is responsible for three tumors, Kaposi sarcoma, multicentric Castleman's disease (CD, a polyclonal B cell hyperplasia) and PEL, monoclonal body cavity lymphoma of the B cell series. Each of the three major proliferative syndromes associated with HHV-8 has a partially different virus expression program.

Only the viral ORF73-coded nuclear antigen, LANA1, is regularly expressed in all three HHV-8 related neoplasms. LANA1 binds to the histone H-1 which tethers the viral DNA to the chromosomes during mitosis, securing the maintainance of HHV-8 episomal DNA in infected cells and the delivery of the viral progeny to the daughter cells. LANA1 is a highly immunogenic protein, expressed predominantly by latent virus in KS cells and in PEL lines. LANA1 represses p53 transcription in a highly specific fashion (Friborg *et al.*, 1999).

The viral cyclin D homologue, K cyclin is expressed from the same polycistronic transcript as LANA1. It can promote apoptosis and growth arrest (Verschueren *et al.*, 2002). DNA synthesis, but not mitosis, continues, leading to multinucleation and polyploidy. Centrosome amplification leads to aneuploidy. In the absence of functional p53, aneuploid cells survive and expand. K cyclin expression in p53−/− and also in wild-type mouse embryonic fibroblasts induces massive centrosome amplification with multiple spindles and fusion bridges. In p53 knockout mice, viral K cyclin induces lymphomas.

Similarly to the small DNA tumor viruses, HHV-8 impairs both the p53 and the Rb pathway. But, unlike the small DNA tumor viruses, HHV-8 has developed multiple tactics to corrupt Rb functions. The viral cyclin K which is, like LANA1, constitutively transcribed in HHV-8 carrying cell lines, inactivates Rb and activates EF2 related transcription (Radkov *et al.*, 2000). LANA 1 and Rb coexist as a complex in vivo. Similarly to adenovirus E1A, papillomavirus E7 and SV-40LT, LANA 1 targets the hypophosphorylated, active form of Rb. Moreover, LANA 1 can overcome the flat cell phenotype, induced by the Rb protein in SAOS2 cells. In cooperation with ras, LANA 1 transforms primary REF cells and renders them tumorigenic. While LANA 1 thus resembles SV40LT in being able to interfere with both Rb and p53 function, its ability to inactivate the two main tumor suppressor pathways is not as complete as the action of LT, because LANA 1 cannot transform by itself.

Double HHV8/EBV carrying PEL cells

The majority of the body cavity (PEL) derived lymphoma lines carry both viruses while some carry only HHV-8. EBV expresses its minimal (type I) program, characteristic for BL. The growth transformation associated, proliferation driving genes, characteristic for the type III program of the LCLs, are not expressed, indicating that the double virus carrier lines are not driven by EBV. EBV may have a similar accessory function as it has in BL.

But are the PEL lines, whether single or double virus carriers, driven by HHV-8? This is a reasonable possibility, but it cannot be taken for granted. Body cavity lymphoma lines have numerous chromosomal changes. Some of them may be responsible for the proliferative activity. There is no single, common cytogenetic change like the Ig/myc translocations in BL. Multiple rearrangements may cover more specific changes, however, as in the cryptic Ig/myc translocations that often hide within the massively rearranged karyotypes of cell lines derived from human multiple myeloma, only detectable by the SKY technique (Gratama *et al.*, 1988).

If HHV-8 occupies the driver's seat in the body cavity lymhomas, RNAi or antisense techniques directed against either LANA1 or viral cyclin K should cause growth arrest or apoptosis. This experiment could go a long way in clarifying the role of the virus in PEL. In the absence of direct transformation systems, such evidence may be quite crucial.

Other HHV-8 genes that may be directly relevant for tumorigenesis include the pirated MIR1 and 2 genes, encoded by K3 and K5. They inhibit MHC class I surface expression, through a unique mechanism not found in other viruses. They remove the MHC I proteins from the plasma membrane by enhanced endocytosis. V-FLIP,

another pirated gene, is a powerful inhibitor of receptor mediated apoptosis. It is expressed constitutively on latent transcripts and it enhances the tumorigenicity of mouse lymphomas. In addition to the crippling of Rb and p53, and the virally encoded bcl-2, v-FLIP may provide additional protection against apoptosis.

Conclusions

EBV and HHV-8 associated malignancies show interesting parallels and contrasts. The main parallel is the universally occurring expression of the viral product required to maintain the viral episomes, EBNA1 in EBV, LANA1 in HHV-8. Both are nuclear proteins, but they act quite differently.

The most prominent and best known feature of EBV is its finely poised adaptation to the B cell compartment and its sophisticated exploitation of B cell biology – in different ways – for viral expansion, the control of that expansion, and for the latency of the persisting virus. It uses a B-cell surface receptor, CD21, as its receptor. It stimulates the infected B-cells to blast transformation, using or mimicking the normal immunoblastic transformation pathways, and it "immortalizes" its targets, driving them to continuous expansion in vivo and in vitro. The immune response of the host, directed against one of the immunogenic, growth transformation associated proteins (the choice depending on the MHC constitution of the host), rejects the expanded blasts, but not before they have substantially raised the viral load. Meanwhile, the virus secures its lifelong latency by downregulating its proliferation driving and its immunogenic proteins (the two categories overlap partly but not completely) and establishes permanent residence in long-lived, post-germinal center memory cells. Since successful bone marrow transplantation eradicates the resident virus population (Damania *et al.*, 2000), all the persisting virus must reside in the hemopoetic compartment, and probably in B-cells.

We have no knowledge as yet of host cell gene expression pattern in latently HHV8 infected cells. It is therefore still an open issue whether the HHV8 tumorigenic process develops from a latent infection, from infection of "non-physiological" host cells or the abortion of a lytic infection. Several of the HHV8 proteins that have not yet been detected in tumors are interesting candidates in relation to tumor risks. Conceivably, the patterns of viral gene expression may differ in precursor cells of different phenotypes.

Interestingly, motifs of the two membrane proteins K1 and K15 mimic several features of LMP 2 a and LMP 1, which are frequently expressed in EBV-associated tumors. They contain potential NFkB- and ITAM-interacting motifs (Damania *et al.*, 2000; Nicholas, 2003). In contrast to the smaller DNA tumor viruses, they may be less in need to control their long term persistence in the host cell and the fate of the host cell through long periods of time. On the other hand, that may be more dependent on modulators of the acute immune response and intracellular controls of proliferation and apoptosis, as part of their lytic virus replication.

Neither the proliferative, nor the resident phase of the viral life cycle requires crippling of the two main tumor suppressor pathways, Rb and p53. The situation is different in EBV carrying malignancies that are not driven to proliferate by EBV (like BL and NPC), but where the virus is present in an adjuvant capacity, probably as an accessory antiapoptotic device.

Most and perhaps all malignant tumors need to inactivate both the Rb and the p53 pathways. This can happen by a number of alternative mechanisms. In BL, the p53 pathway can be crippled by p53 mutation/deletion, ARF mutation or MDM2 amplification. The Rb pathway can be inactivated by Rb mutation/deletion, p16 inactivation or CDK4 amplification. There is no reason why the minimal (type I) EBV program expressed in BL cells should interfere with the Rb or the p53 pathway and there is no evidence that it does.

In HHV-8 associated tumors, the situation is quite different. In the absence of an in vitro transforming system, we cannot tell whether any of the viral genes (including the genes pirated from the host cell) can drive cell proliferation. We know, however, that two of the genes, LANA1, and viral cyclin K cripple the Rb and p53 pathways. This brings HHV-8 into the previously established DNA tumor virus fold.

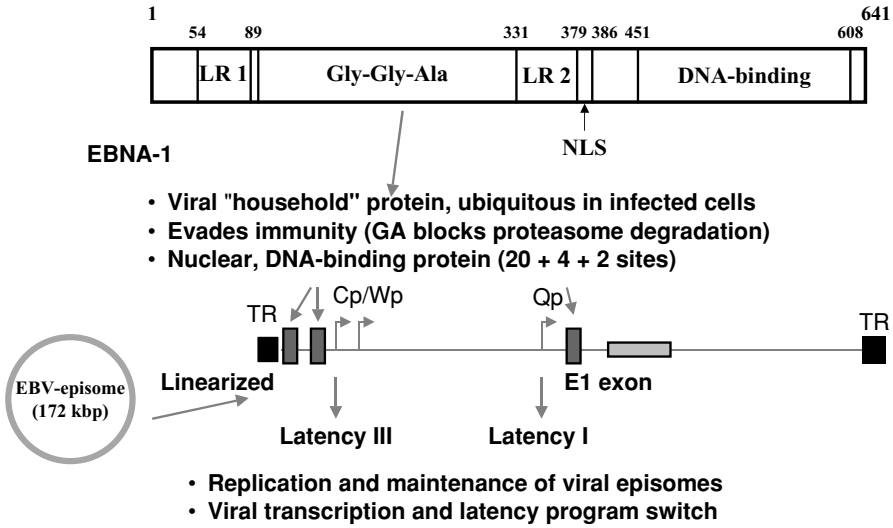


Fig. 29.1. Schematic representation of EBNA 1.

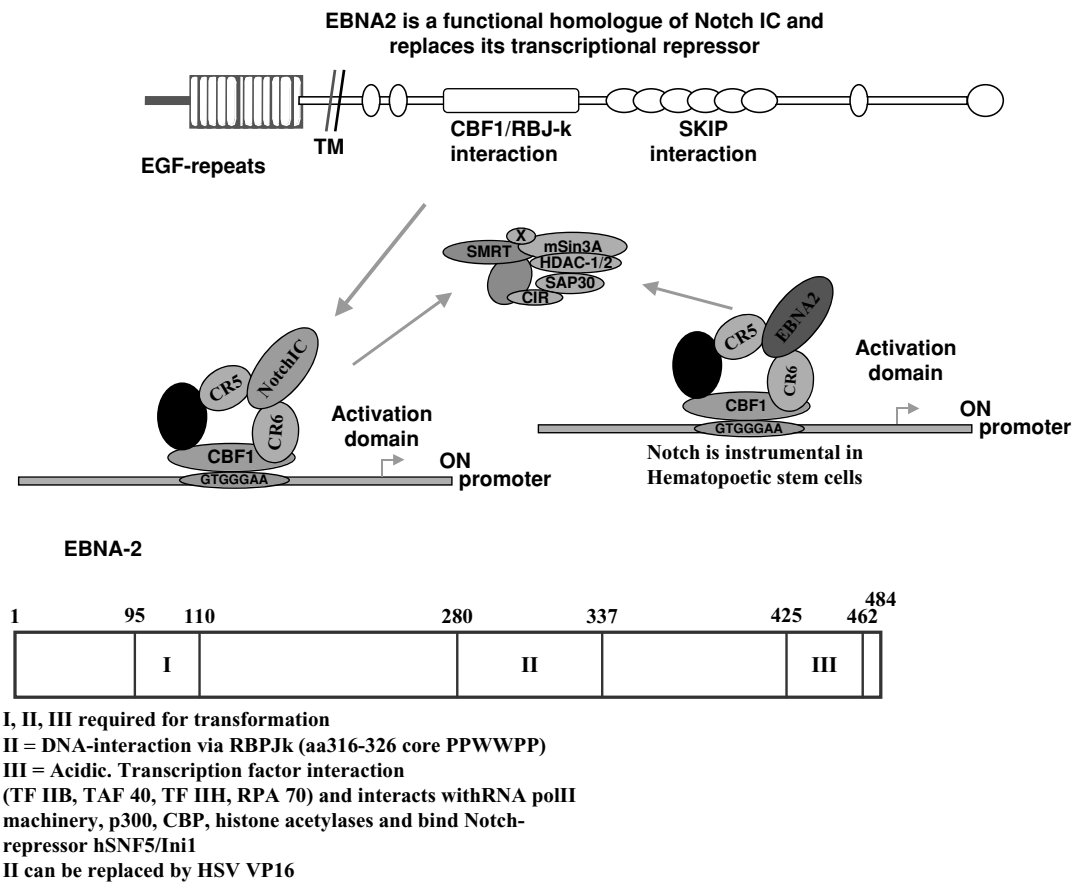


Fig. 29.2. Schematic representation of EBNA 2 and its major functions.

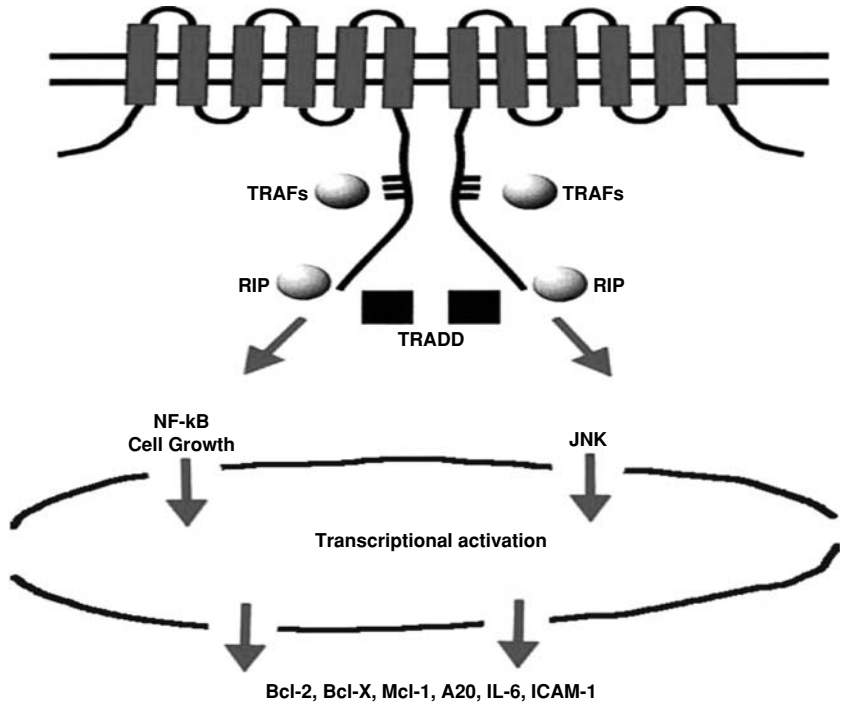
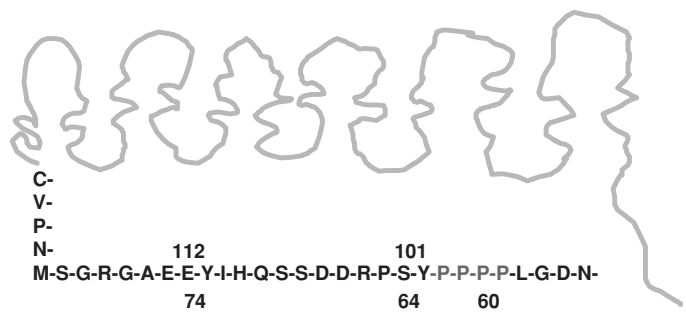


Fig. 29.3. Established major signaling pathways of LMP 1. Studies have revealed several functional domains of the cytoplasmic tail of LMP1 that are important for activation of transcription factor NF-kB, AP-1 and P38 through its interaction with TRAF and TRADD. These findings suggest that LMP1 mimics TNFR aggregation, which is essential for subsequent signal transduction.

The N-terminus of EBV-LMP2a contains eight phosphotyrosine motifs and two PPPPY-motifs



C-
V-
P-
N-
112 101
M-S-G-R-G-A-E-E-Y-I-H-Q-S-S-D-D-R-P-S-Y-P-P-P-P-L-G-D-N-
85 74 64 60
G-D-H-Q-L-G-L-Y-L-S-Q-D-Q-T-G-P-Q-Y-D-S-H-R-D-G-N-G-W-Y-P-D-E-Y-P-P-P-P-E-
31 23
E-N-S-E-R-E-E-D-N-P-P-T-P-T-N-G-S-S-G-A-S-P-Y-Q-S-N-N-G-G-D-Y-G-D-P-D-G-G-
P-S-P-P-G-A-G-M-P-V-M-E-L-S-G-M

EBV-LMP2a eight phosphotyrosine motifs interact with SH2- and WW-domain kinases, adaptors and ubiquitin-pathway components

Aa pos	Motif	Associated proteins
112	A-E-E-Y	Lyn, Fyn, Lck, Src, Blk, Src family PTKs
101	R-P-S-Y-P-P-P-P	Csk, WW-domain (E3-ubiquitin)
85	L-G-L-Y	Syk (ITAM:YXXL)
74	L-P-Q-Y	Syk (ITAM:YXXL)
64	N-G-W-Y	None?
60	Y-P-D-E-Y-P-P-P-P	Abl, Crk, Nck, WW-domain (E3-ubiquitin)
31	A-S-P-Y	Shc, PI-3-kinase, PLCgamma2?
23	G-G-D-Y	None?

Fig. 29.4. N-terminal phosphotyrosine motifs of LMP 2 a.

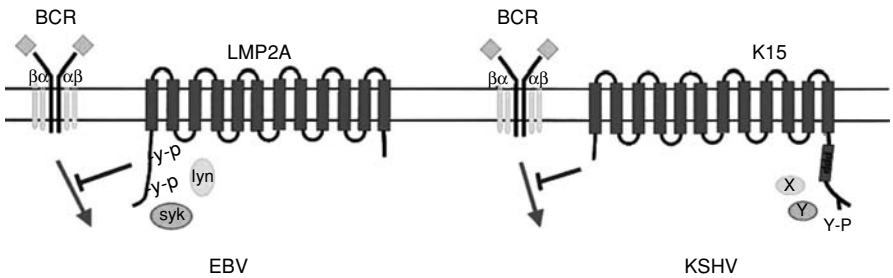


Fig. 29.5. Functional comparison between the Epstein–Barr virus LMP2a and the Kaposi Sarcoma virus (HHV8) K1 and K15 membrane proteins.

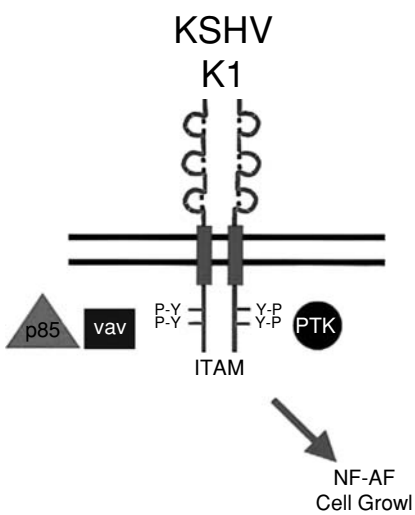


Fig. 29.6. HHV 8 K1 membrane protein.

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30

KSHV manipulation of the cell cycle and apoptosis

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Disruptions of cell cycle and apoptotic regulatory control are primary hallmarks tumor cells. It is therefore not surprising that Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8), a tumor virus, encodes viral proteins targeting these cell growth regulation mechanisms. The extent and range of KSHV genes devoted to manipulating these processes is, however, remarkable.

As described in previous chapters, herpesviral structural and replication-related genes are highly conserved among the herpesviruses, including KSHV. In contrast, regulatory genes generating proteins that modify the cellular environment – particularly during latency – are generally unique to each virus. As will become evident in this chapter, even though KSHV and EBV are closely related to each other, there are few sequence homologies among the oncogenes and non-structural regulatory genes found in the two viruses. Despite this, there is a striking functional similarity between the two viruses (Table 30.1). EBV encodes multiple highly evolved transcription factors and signaling proteins that induce many of the same cellular genes that KSHV has pirated into its genome. Further, once herpesvirus targeting of a cellular pathway has been found for one herpesvirus (e.g., HSV-1 downregulation of MHC I surface expression, Hill *et al.*, 1994), searching for functional similarities among other herpesviruses has been particularly rewarding (e.g., Coscoy *et al.*, 2000). It is therefore not surprising that KSHV and EBV share pathways for cell transformation although they achieve this through very different mechanisms.

Several general principles for KSHV regulatory gene functions that affect cell transformation and tumorigenesis can be made with the important caveat that exceptions exist to each rule.

1. Although there is little or no sequence homology among oncogenes encoded by different tumor viruses, the cellular targets for viral oncogenes are frequently conserved (Table 30.1). For example, direct inhibitors of p53 from KSHV, adenoviruses, polyomaviruses, and papillomaviruses have no apparent similarity to each other but all of these viral proteins target and inhibit p53 functions.
2. KSHV proteins encoded by viral genes pirated from the cellular genome have similar functions to their cellular counterparts. They generally differ in their regulation rather than their function. For example, the KSHV vCYC protein acts as D-type cyclin in cells but unlike cellular cyclins it is resistant to inhibition by cyclin-dependent kinase inhibitors but are modified to escape normal cellular regulation. The evolution of entirely new KSHV gene functions is uncommon and differences between cellular and KSHV homologues lie primarily in their expression and regulation (for an exception, however, see description of ORF36 mRNA shut-off functions in Chapter 56).
3. KSHV targeting of cellular tumor suppressor pathways also inhibits host defenses against viral infection which indeed may be the principle benefit to the virus for these proteins (Moore & Chang, 1996, see also Chapter 31). Signaling pathways controlling of tumor cell growth, such as Fas-FasL death receptor signaling, also critical for both innate and adaptive immune responses, and are targeted by putative KSHV oncoproteins.

This chapter describes the effects of individual KSHV proteins on cell cycle control, apoptosis, and cellular transformation. It is, by necessity, an artificial division of these functions, since cell growth control mechanisms are intimately tied into other aspects of the viral lifecycle such as maintenance of latency (Chapter 24) and immunoevasion (Chapter 31). Caution is warranted given the limitations to our ability to study how KSHV contributes to cancer. Our understanding of viral effects on cell growth are generally based on in vitro cell culture systems, e.g., PEL cell

cultures, and on the study of individual genes in isolation. Much can be learned from this reductionist approach, but it is important to keep in mind that results from the bench-top do not always translate to the bedside, which is more fully addressed in Chapters 50 and 56).

Cell cycle and programmed cell death regulation

The normal cell cycle is actively regulated through specific protein-kinase regulatory subunits, cyclins (Murry, 2004), that are cyclicly expressed during the different phases of the cell cycle and degrade as the cell exits each phase (Fig. 30.1). Cyclins regulate the periodic oscillations of the cell cycle by coupling to cyclin-dependent kinases (which tend to be constitutively expressed) to phosphorylate specific cell cycle regulatory targets, for example, the retinoblastoma protein (pRB1) controlling expression of genes necessary for transit from G1 into S phase. The cyclin component of the dimeric complex generally serves as the targeting moiety directing CDK phosphorylation to specific substrates.

Some cyclins such as cyclin A and the D-type cyclins do not appear to be essential for intrinsic cell cycle periodicity but serve to regulate it (Kozar *et al.*, 2004). pRB1 (a member of a family of related proteins that also includes p130) is a transcriptional repressor that binds and inactivates E2F family transcription factors involved in transcription of genes required for DNA synthesis prior to S phase. These enzymes include dihydrofolate reductase, thymidine kinase, and several other nucleotide synthesis enzymes expressed during the G1/S phase transition and have been pirated by KSHV (Russo *et al.*, 1996). When pRB1 is hyperphosphorylated by cyclin D-CDK4 or CDK6, it is inactivated and releases E2F allowing expression of DNA synthesis enzymes. Thus pRB1 is a critical regulator for the transition between G1 and S phase and controls the cellular environment to prevent unscheduled DNA synthesis. Another substrate phosphorylated by cyclin-CDK complexes is the anaphase-promoting complex (APC), which controls chromosome separation during mitosis and acts as a checkpoint protein at the G2/M cell cycle transition.

Viral manipulation of cell cycle checkpoint proteins could be an obvious advantage to a virus, particularly during lytic viral replication when large amounts of viral DNA must be generated. But, interference with normal cell cycle regulatory circuits often activates cell cycle arrest or programmed cell death (apoptosis) signaling. Cell cycle and apoptotic pathways are characterized by extensive feedback interactions that not only serve to prevent tumor cell generation but also to inhibit viral replication within cells (Takaoka *et al.*, 2003). Viral inhibition of pRB1, for example, results in p53 activation through p14ARF and MDM2 (Fig. 30.1). Thus, overexpression of individual cellular or viral oncoproteins may paradoxically result in cell cycle arrest or cell death rather than proliferation, as is the case for cellular cMYC, or adenoviral E1A (de Stanchina *et al.*, 1998, Debbas and White, 1993; Zindy *et al.*, 1998). Because of this feedback regulation, viral proteins that initiate dysregulated cell cycle entry can be mistaken to have the exact opposite effect, e.g., cell cycle arrest or apoptosis. Caution must be used to interpret the consequences of viral protein expression in the context of the actual viral lifecycle in which multiple KSHV proteins may be acting in concert.

Table 30.1. Functional similarities between KSHV, EBV and other tumor viruses

	KSHV	EBV	Other viruses
pRB1 inactivation	vCYC	EBNA2/EBNA-LP	Ad, HPV, SV40
β-catenin stabilization	LANA1	LMP1	
p53 inhibition	LANA1, vIRF1, LANA2	LMP1	Ad, HPV, SV40
Anti-apoptotic, NF-κB activation	vBCL2, vIAP, vFLIP, LMP1	BHRF1, LMP1	Many viral proteins
BCR signaling	ORFK1, LAMP	LMP2	?
Death receptor inhibiton	vFLIP	LMP1	HVS, MCV
CBP/p300 inactivation,	vIRF1	EBNA2	Ad, CMV, HTLV I,
Interferon transcription factor inhibition	ORF45 protein		HPV, SV40
MHC down-regulation	MIR1/2	–	Ad, HSV, CMV
IL-6 signaling	vIL-6	LMP1	?
Telomerase activation	LANA1	?	HPV
Th2 Chemokines	vMIP 1-3	?	CMV

Cell cycle dysregulation during viral latency

LANA-1

Although lytic KSHV replication may contribute to tumor formation in humans though paracrine mechanisms (see Chapter 56), most interest is focused on the latent viral genes constitutively expressed in tumor cells as oncogenes. Whereas latent virus replication is compatible with cell expansion, virion production generally leads to cell death. As described in Chapter 28, the division between "latent" genes and "lytic" genes, however, has become increasingly blurred. Intracellular notch (Chang *et al.*, 2005) or interferon signalling (Chatterjee *et al.*, 2002) can activate expression of genes such as K1 (KIS) and K2 (vIL-6) that are traditionally referred to as lytic genes without full lytic cascade

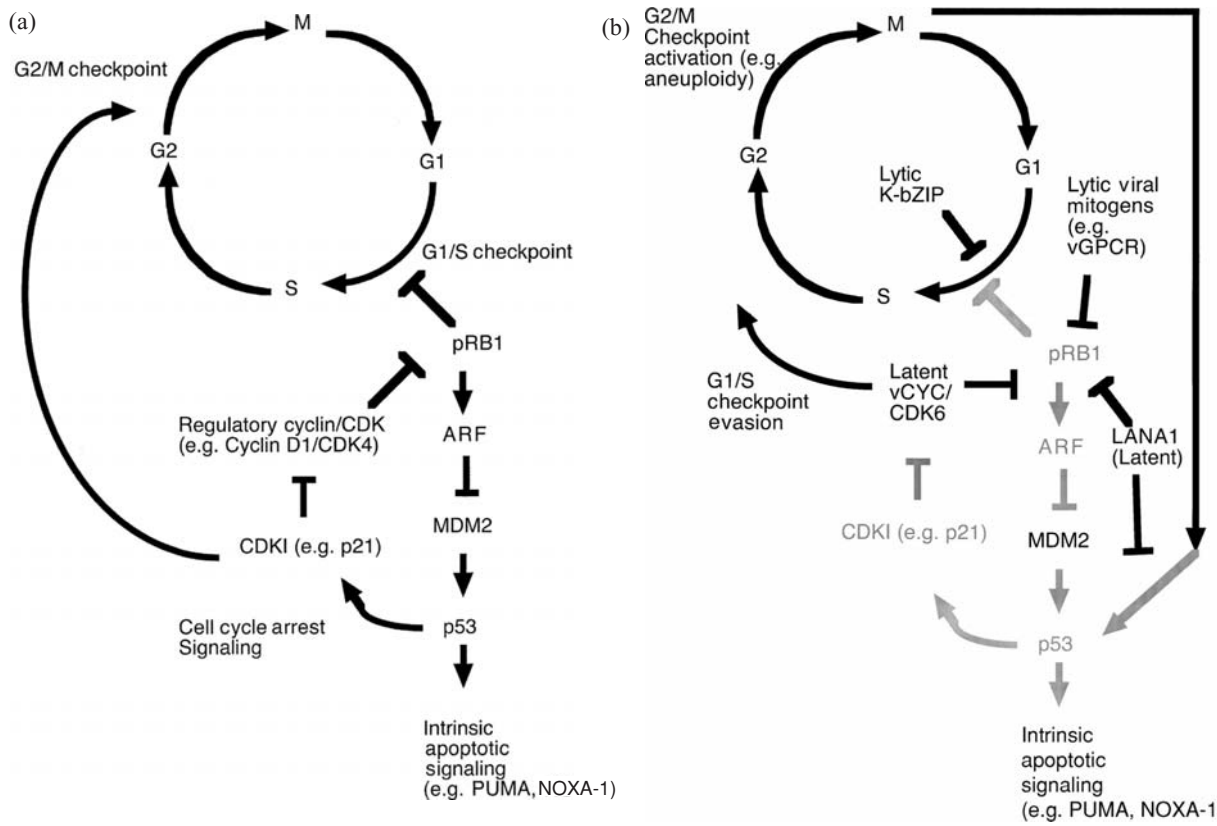

Fig. 30.1. A schematic diagram of cell cycle control interactions between the G1/S checkpoint retinoblastoma protein (pRB1) and p53. (a) Normal feedback control allows pRB1 to negatively regulate entry into the S phase by inhibition of E2F-regulated DNA synthesis genes and positively regulate p53 activity through ARF-dependent inhibition of the p53 E3-ligase, MDM2. Mitogenic cyclins such as cyclin D bind to cyclin-dependent kinases that than phosphorylate and inactivate pRB1, allowing S phase entry. Uncontrolled activation of this circuit will induce p53 activity to initiate cell cycle arrest or apoptosis. KSHV vCYC and LANA1 proteins act together during latent viral replication to inhibit both the pRB1 control functions and p53-dependent apoptosis. Inhibition of p53 may be particularly important since dysregulated entry into the cell cycle by vCYC can initiate aneuploidy and dysregulated cytokineses. During lytic replication a more complicated pattern arises since KSHV proteins can both block (K-bZIP) and enhance (vGPCR, vIL-6) G1/S cell cycle transit, to enhance virion production.

activation and cell death. Notch signaling in particular appears to be responsible for the Type II pattern of KSHV gene activation (Sarid *et al.*, 1998), markedly increasing the number and range of KSHV genes having potential to play a role in KSHV-induced tumorigenesis. Intriguingly, the latent antigen LANA1 has been reported to activate notch signaling and intracellular notch activity is high in resting PEL cells (Lan *et al.*, 2006).

LANA

The major latency locus on the right-hand end of the genome (Fig. 30.2) encodes three open reading frames (ORFs K13, 72 and 73) for the vFLIP, vCYC and LANA1 proteins. An additional long transcript expressed by polyadenylation site read-through from this promoter is processed into at a number of miRNAs that are also constitutively expressed (Pfeffer *et al.*, 2005; Cai *et al.*, 2005). While the viral or cellular targets for these miRNAs are unknown, they are of particular interest since cellular miRNAs have been closely associated with cancer cell regulation (Esquela-Kerscher and Slack, 2006). Recent studies also reveal the complexity of gene expression at this locus with some transcripts extending to the K12 region. Some overlapping transcripts for ORFs K12, K13, 72 and 73 have been shown to be induced during lytic replication demonstrating that the widely-held view that these genes are expressed in a static fashion is incomplete (Cai and Cullin, 2005; Pearce *et al.*, 2005).

ORF73 encodes LANA1 (Latency-associated nuclear antigen)1, first discovered as a serologic antigen useful for

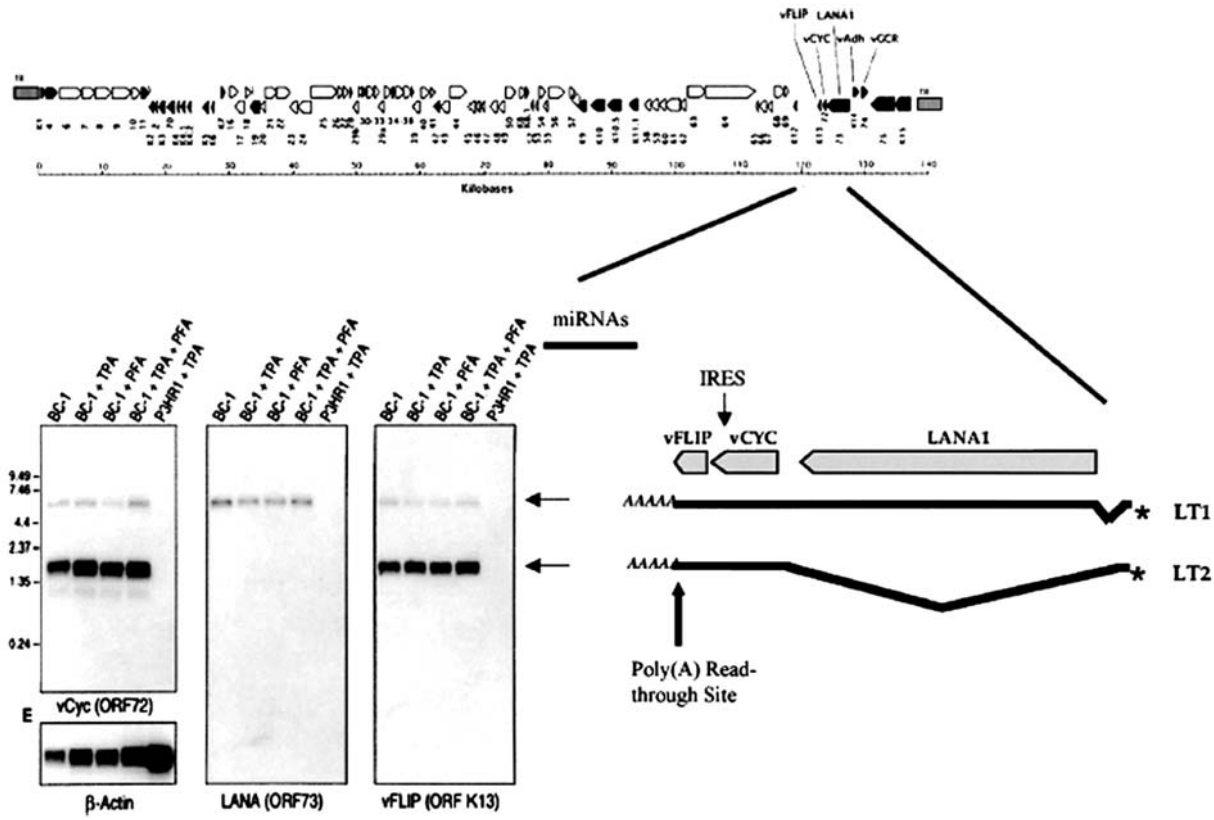

Fig. 30.2. Diagram of the KSHV major latency locus. Northern blots are shown for the major genes in this region under conditions of lytic induction (TPA) or viral DNA synthesis inhibition using the DNA polymerase inhibitor, phosphonoformic acid (PFA). The genes encoding LANA1, vCYC and vFLIP are expressed in the BC-1 cell line under all conditions, but they are also under cell cycle regulation. Less abundant transcripts, including transcripts expression KSHV miRNAs after read-through of the LT1/LT2 polyadenylation site, are not seen at this exposure. (From Sarid *et al.*, 1999.)

Table 30.2. Major KSHV proteins affecting cell cycle control machinery

	Protein	Gene	Target(s)
Latency	LANA1	ORF73	pRB1
	vCYC	ORF72	pRB1, p27, ORC1, H1, Cdc25a
Lytic or Induced	vGPCR	ORF74	Akt, SAPK pathways
	KIS	ORF K1	ITAM signaling
	vIL-6	ORF K2	Interferon, IL-6 signaling
	vMIP 1–3	ORFs K4, 4.1 and 6	Angiogenesis
Lytic	K-bZIP (RAP1)	ORF K8	C/EBP-α, p21

detecting KSHV infection (Moore *et al.*, 1996), that functions to maintain the viral episome by tethering it to cellular chromosomes during mitosis (Ballestas *et al.*, 1999, see Chapter 24). LANA is mainly expressed from a polycistronic transcript (LT1) that includes vCYC and vFLIP (Figure 30.2). These two latter proteins are also expressed from a second transcript (LT2) that originates from the same promoter but splices out ORF73. The latency promoter is cell cycle-regulated with highest expression during late G1 (Sarid *et al.*, 1998) and LANA1 positively autoregulates its own expression as well (Jeong, *et al.*, 2004). Although LANA1 and vCYC proteins are translated from LT1 and LT2 respectively, vFLIP translation requires an internal ribosomal entry site (IRES) located at the 3' end of the vCYC message (Grundhoff and Ganem, 2001; Bieleski and Talbot, 2001; Low *et al.*, 2001). Outside of the major latency locus, the ORF K10.5 gene product, vIRF3 or LANA2, is also constitutively expressed in KSHV-infected hematopoeitic cells but not KS

tumor cells (Cunningham *et al.*, 2003). Other KSHV genes, including K12 (kaposin) are expressed during latency but are also induced during lytic replication and by other transcriptional activation, such as notch-signaling (Chang *et al.*, 2005).

LANA1 is a large protein (*ca.* 150 kDa) composed of basic, glutamine-rich amino- and carboxyl-terminal domains separated by a highly acidic, aspartic- and glutamic acid-rich repeat region that includes a leucine zipper domain (see Chapter 24). Because of its highly charged, amphipathic structure it runs as a 220–224 kDa doublet on denaturing gel electrophoresis. This multifunctional protein is reminiscent of the SV40 virus large T-antigen (LT ag), since it tethers the episome to cellular DNA during mitosis and also disrupts both cell cycle and p53-mediated apoptosis.

LANA1 abrogates cell cycle arrest through its binding to at least two of the many cellular interaction partners for LANA1 that have been described. Radkov and colleagues (Radkov *et al.*, 2000) found that LANA1 directly binds the hypophosphorylated (active) form of RB1, inhibiting RB1's ability to serve as a transcriptional repressor of the E2F family. LANA1 binds the pocket region of RB1, but not the related RB1-like protein p130, through interactions with a central region that includes the leucine zipper domain. This effectively sequesters RB1, allowing E2F transactivation (Figure 30.1). Dysregulation of the G1/S checkpoint by LANA1 can be functionally demonstrated rodent cell transformation assays. While LANA1 expression alone does not enhance cellular transformation, oncogenic cooperativity occurs when LANA1 is coexpressed with the activated H-Ras oncogene.

Aside from direct inhibition of RB1, LANA1 as a second pro-mitogenic activity through its ability to activate the Wnt signaling pathway. Hayward and colleagues identified glycogen synthase kinase – 3β (GSK-3β) as a protein interactor with LANA1 through yeast two-hybrid screening (Fujimuro and Hayward, 2003; Fujimuro *et al.*, 2003). GSK3-β normally phosphorylates β-catenin causing the cytoplasmic sequestration of this proto-oncoprotein. LANA1, however, inhibits GSK-3β regulation of β-catenin allowing it to accumulate in the nucleus and transactivate responsive promoters, including the cMYC promoter. cMYC has diverse mitogenic activity and activates the hTERT promoter, which may contribute to the ability LANA1 to activate telomerase activity in KSHV infected cell lines (Verma *et al.*, 2004; Wu *et al.*, 1999). GSK3-β inhibition could potentially also indirectly modulate cell cycle entry through its ability to phosphorylate D-type cyclins resulting in their cytoplasmic accumulation (Verschuren *et al.*, 2004b). These secondary consequences of LANA targeting of Wnt pathway signaling are speculative and remain to be examined but suggest novel and unexplored ways that LANA can contribute to cell transformation. As is the case for many KSHV signaling pathways, EBV has also been shown to stabilize β-catenin showing conservation of cell signaling pathway targeting by the two vriuses (Hayward *et al.*, 2006).

Viral Cyclin (vCYC)

While LANA1 has no homologue in the human genome, KSHV also uses its pirated cyclin to hijack cell cycle regulatory control mechanisms. The vCYC protein (ORF72) (Cesarman *et al.*, 1996; Chang *et al.*, 1996) is expressed together with LANA1 from the major latency locus. As previously described, cellular cyclins positively regulate various stages of the cell cycle by partnering with CDKs to phosphorylate specific cell cycle components. KSHV vCYC retains sequence similarity to the D-type cyclins (there are three cellular D cyclins-D1, D2, and D3 which have overlapping and apparently redundant activities), which target CDK4 and CDK6 to hyperphosphorylate RB1, thereby inhibiting this inhibitor of E2F-induced transcription. KSHV vCYC partners almost exclusively with CDK6 to achieve this effect (Chang *et al.*, 1996; Godden-Kent *et al.*, 1997; Li *et al.*, 1997), initiating illicit S phase entry and DNA replication (Laman *et al.*, 2001).

Although vCYC is structurally similar to D-type cyclins, several features of the protein are unique. Unlike D-type cyclins, vCYC (also referred to as K-cyclin) targets cell cycle control proteins that are more typical targets of other cyclins such as cyclins A and E which couple to the CDK2 kinase. These targets include histone H1, the CDKI p27, and Cdc25a, as well as ORC1 and Cdc6 (Ellis *et al.*, 1999; Laman *et al.*, 2001; Mann *et al.*, 1999) (for review see Verschuren *et al.*, 2004b). Unlike cellular cyclin D/CDK6 complex, which normally requires CDK6 phosphorylation through the action of a CDK-acitvating kinase (CAK), the vCYC/CDK6 is fully active in an unphosphorylated form (Child and Mann, 2001; Kaldis *et al.*, 2001).

The vCYC gene, ORF72, like the other genes encoded in the major latency locus, is expressed during late G1 in a cell cycle-dependent fashion (Sarid *et al.*, 1999). Although direct measurement of vCYC protein levels are difficult, vCYC lacks the cyclin destruction box motif that targets cyclins for rapid protein turnover (Klotzbucher *et al.*, 1996) suggesting that the viral protein may be long-lived and active at other portions of the cell cycle including the G2/M checkpoint (Van Dross *et al.*, 2005). Also absent from the viral protein are motifs required for docking to nuclear export proteins, which may indicate that vCYC abnormally accumulates in the nucleas and may escape regulations imposed on cellular cyclin proteins (Verschuren *et al.*,

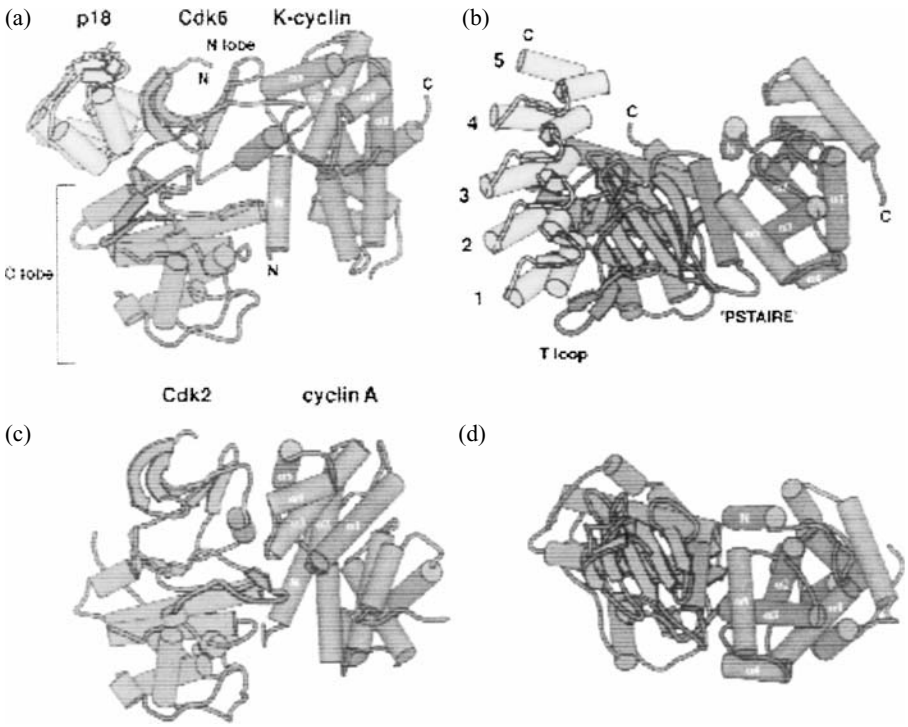

Fig. 30.3. The structure of the vCYC (purple), CDK6 (cyan), p18Inkb (yellow) complex from side (a) and top (b) views, compared to cellular cyclin A (purple), CDK2 (cyan) side (c) and top (d) views. Unlike cellular cyclins, the regulatory T-loop of CDK6 is excluded from interaction with vCYC but the PSTAIRE regulatory helix of CDK6 still forms an interface with vCYC. The PSTAIRE helix forms part of the ATP binding domain required for kinase activity while the T loop acts as a negative regulator of kinase activity and must be phosphorylated by cyclin-activating kinases (CAK) in cellular cyclin–CDK complexes. While CAK phosphorylation may enhance vCYC-CDK6 stability, displacement of the T loop by vCYC allows this complex to be active in the absence of CAK activity. The structure of vCYC-CDK6 also reveals loss of the binding pocket used by cyclin-dependent kinase inhibitors (CDKI) of the CIP1/KIP1 family. These and other features support experimental data showing the vCYC-CDK6 not only have a broader target range than cellular D-type cyclins but also escape many normal negative regulatory controls imposed on the cellular cyclin machinery. Reprinted with permission (Jeffrey *et al.*, 2000). (See color plate section.)

2004b). This is supported by transgenic mouse experiments (see below) in which constitutive vCYC expression leads to defects in chromosome segregation, abnormal cytokinesis and polyploidy (Verschuren *et al.*, 2002). Surprisingly, vCYC also phosphorylates and inactivates the antiapoptotic cellular protein BCL-2, but not the corresponding KSHV BCL-2 homologue, contributing to the pro-apoptotic properties of this protein when overexpressed in cells (Ojala *et al.*, 2000).

Another critical difference between cellular cyclins and vCYC is that vCYC escapes from normal CDK inhibitor (CDKI) control. CDKIs, including p21 and p27 and members of the p16Ink4a family, act to inhibit cyclin-dependent phosphorylation and are a critical link for transmitting cell arrest signaling from p53 to the cell cycle machinery. vCYC/CDK6 is resistant to p21 and p27 CDKI inhibition (Swanton *et al.*, 1997, 1999), but is still inhibited by Ink4 unless CDK6 is phosphorylated through the action of CAK (Jeffrey *et al.*, 2000). This has allowed crystallographic analysis of the trimolecular vCYC/CDK6/p18Inkb structure (Fig. 30.3), which has been tremendously informative for understanding the structural basis for vCYC regulation as well as regulation of the cellular cyclins. vCYC phosphorylation of p27 also diminishes cellular levels of this inhibitor protein, contributing to positive cell cycle regulation. In summary, although vCYC has similar functionality to cellular cyclins, modifications to this protein allow the virus to escape normal cell cycle regulatory constraints and to couple CDK6 phosphorylation to RB1 and other cell cycle components (for review, see Mittnacht and Boshoff, 2000; Verschuren *et al.*, 2004b).

A potential role for vCYC in cell tranformation and carcinogenesis has been uncovered using transgenic mice in which vCYC is expressed under an Eμ promoter (Verschuren *et al.*, 2002, 2004a). Overexpression of vCYC both

induces dysregulated DNA synthesis and leads to p53-dependent apoptosis. One mechanism by which this could occur is a circuit in which RB1 inactivation leads to E2F activation of p14ARF – a potent activator of p53 acting through MDM2. This, however, does not seem to be the case since p53-induced apoptosis is not reduced by mating the transgenic vCYC mice onto a p19ARF-null background. Instead, vCYC appears to dysregulate the G2/M checkpoint resulting in dysregulated cytokinesis and aneuploidy that in turn activates p53 through DNA-damage response pathways. When mice expressing vCYC are mated with p53-null mice, progeny develop lymphomas at an accelerated rate.

Other KSHV mitogenic signaling proteins

KSHV also possesses a number of proteins which act through mitogenic signaling pathways to achieve similar effects. Mitogenic signaling can occur through diverse signaling pathways which act on common regulatory points to induce cell cycle entry, such as activation of cyclin-dependent phosphorylation of RB1 (Sherr, 2004). While most of these molecules are thought to be expressed only during active lytic replication, evidence suggests that they have more complicated patterns of expression (see Chapter 28) in that at least several of them, such as the vIL-6 and the KSHV-encoded chemokines (Moore *et al.*, 1996; Parravicini *et al.*, 2000), are expressed at low levels during true viral latency (at least in tissue culture where the process can be readily examined) and are further activated during lytic viral replication or in response to specific signaling pathways. This is a similar pattern of expression to the EBV LMP1 protein. It is unclear what, if any, effects these mitogenic factors have during latency but these pathways have also received renewed interest related to the possibility that lytic virus replication contributes to the tumorigenesis, particularly in KS tumors (described more fully elsewhere in this volume).

Viral G-protein coupled receptor (vGPCR)

The best-studied example of a KSHV mitogenic signaling molecule is the vGPCR (ORF74), a seven-spanning G-protein coupled receptor that is expressed at early phases during lytic replication (Cesarman *et al.*, 1996). vGPCR is a constitutively active CXC receptor (Chiou *et al.*, 2002; Kirshner *et al.*, 1999), which activates MAPK, p38, Akt and NF-κB pathways resulting in expression of angiogenic factors, such as VEGF, and in cell transformation (Bais *et al.*, 1998; Cannon *et al.*, 2003; Masood *et al.*, 2002; Montaner *et al.*, 2001; Polson *et al.*, 2002). Although constitutively active, evidence suggests that its growth promoting activity is enhanced by host ligand binding (Holst *et al.*, 2001). The principal interest in this protein comes from the unique phenotype that it generates in transgenic mice which was first demonstrated by Lira and colleagues (Yang *et al.*, 2000) but has been confirmed using a variety of experimental systems by others (Guo *et al.*, 2003; Montaner *et al.*, 2003).

In these first experiments, vGPCR was expressed under control of the CD2 promoter, resulting in diffuse hematopoietic expression of the viral protein. Surprisingly, this resulted in endothelial tumors pathologically resembling Kaposi sarcoma tumors. These and similar results by Montaner and colleagues (Montaner *et al.*, 2003) suggest that KS tumors, unlike primary effusion lymphomas, may actually be dependent on active lytic viral replication and that KS tumor cell proliferation occurs in *trans* due to paracrine factors released by infected cells undergoing lytic replication. This is a unique pathogenic model in which paracrine factors, rather than endogenous genetic changes, are responsible for the neoplastic phenotype (Cesarman *et al.*, 2000).

Several findings, however, complicate this view. It is evident that virtually all tumor cells in KS tumors are infected with KSHV and so paracrine-effects of lytic replication appear to act in concert with endogenous viral gene expression to result in tumor cell outgrowth (Parravicini *et al.*, 2000, Katano *et al.*, 2000). KS development can be effectively prevented by antilytic DNA pol inhibitors, such as ganciclovir (Martin *et al.*, 1999), but there is no current evidence that these drugs have any effect on established KS tumors (Little *et al.*, 2003). Further, clinical studies suggest that some KS tumors arising during transplantation are donor derived (Barozzi *et al.*, 2003). Since these donors were ostensibly healthy, the prototumor was primarily latent, or at least subclinical, in the donor prior to the transplantation. Studies of cell and virus monoclonality do not clarify the origin of KS pathogenesis since both cellular monoclonality and multiclonality have been reported. KS tumors have KSHV terminal repeat patterns that are oligoclonal or monoclonal, with the possibility that tumors evolve into a monoclonal pattern over time (Judde *et al.*, 2000; Russo *et al.*, 1996). The role for paracrine-induced proliferation from vGCPR or other viral proteins may become clearer as KSHV gene regulation outside of the latent-lytic expression pattern is explored.

K1 Protein

On the opposite end of the KSHV genome (see Chapter 28), a second membrane signaling protein encoded by ORFK1, also called KIS (KSHV ITAM signaling protein), has strong mitogenic activity when expressed in cells. K1 protein is a type 1 transmembrane protein that aggregates through ectodomain disulfide bonds into a signaling complex (Lee *et al.*, 1998b). The cytoplasmic tail of the protein contains two immunoreceptor tyrosine signaling motifs (ITAMs) which recruit SH2-containing signaling kinases; NFAT, syk, vav and other downstream signaling effectors have been

shown to be activated by K1, resulting in Akt signaling activation (Lagunoff *et al.*, 1999, 2001; Lee *et al.*, 1998a, 2003; Tomlinson and Damania, 2004, Wang *et al.*, 2005). As a consequence of its mitogenic signaling activities, K1 transforms rodent fibroblasts and primary endothelial cells in vitro and when substituted into a herpesvirus saimiri backbone, induces lymphomas in rhesus macques (Lee *et al.*, 1998b, Wang *et al.*, 2005).

Clues to the functional advantage of this signaling protein for the virus, come from overlap between K1 signaling and signaling through the B cell receptor (BCR). The BCR is active as a non-specific innate immune signaling pathway. Intriguingly, K1 causes ER retention and degradation of the BCR, suggesting that K1 may serve as a decoy molecule after downregulation of this pathway (Lee *et al.*, 2000). A consequence of K1 expression is induction of paracrine angiogenic factors including vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9), analogous to the paracrine mitogenic induction that can occur with vGPCR (Wang *et al.*, 2004). The immunoevasion/mitogenic properties of K1 have analogy, together with the KSHV LAMP1 protein, to properties of EBV membrane proteins, LMP1 and LMP2 (Damania *et al.*, 2000).

Virus-encoded chemokines and cytokines

In addition to both vGPCR and K1 protein, which induce secretion of proliferative cytokines, KSHV itself encodes three secreted chemokines, vCCL1 (*ORF K6*), vCCL2 (*ORF K4*) and vCCL3 (*ORF K4.1*), formerly known as vMIP-I/MIP-1a, vMIP-II/MIP-1b and vMIP-III/BCK, respectively and a functional, secreted vIL-6 (ORF K2) cytokine. The chemokines are believed to act to polarize local immune responses towards a Th2 phenotype. vCCL2 initiates a strong chemotactic response through CCR3 activation (Boshoff *et al.*, 1997), while vCCL1 and vCCL3 activate CCR8 (Dairaghi *et al.*, 1999, Endres *et al.*, 1999) and CCR4 receptors (Stine *et al.*, 2000). All three chemokines have angiogenic activity and stimulate endothelial and B-lymphocytic proliferation (Moore *et al.*, 1996; Stine *et al.*, 2000). While they are generally not expressed in PEL cells during latency, they may play a role in mitogenesis in KS tumors.

Like K1 protein, KSHV encoded vIL-6 has both immune evasion and cell cycle regulatory properties (Moore *et al.*, 1996; Nicholas *et al.*, 1997) (described in more detail in Chapter 31). vIL-6 is a secreted cytokine similar to the human cytokine (25% identity, 62% similarity) that activates IL-6 signaling pathways by binding directly to the gp130 signal transducer molecule without requiring interaction with the IL-6 specific recepor, gp80 (Burger *et al.*, 1998; Chow *et al.*, 2001; Molden *et al.*, 1997; Mullberg *et al.*, 2000; Osborne *et al.*, 1999). Although this has not been directly examined, it is likely that the viral protein activates the same signaling pathways as hIL-6, resulting in RB1 hyperphosphorylation and mitogenesis (Urashima *et al.*, 1996, 1997; Zhu *et al.*, 1994). Studies of a non-adapted tissue culture PEL cell line, BCP-1, demonstrate that the cells are autocrine dependent on hIL-10 and vIL-6 (Jones *et al.*, 1999), and can reinitiate DNA synthesis for serum-starved PEL cells (Chatterjee *et al.*, 2002).

The role of vIL-6 in the lifecycle of KSHV reveals the intimate connection between cell cycle regulation and early innate immune responses (Chatterjee *et al.*, 2002). Type I interferon signaling in PEL cells causes upregulation of p21 CDKI and initiates arrest. The vIL-6 promoter, however, has transcription elements responsive to interferon signaling and is simultaneously up-regulated. Evidence suggests that vIL-6 can block interferon signaling at the receptor resulting in a negative feedback loop that protects infected cells from cell-cycle arrest effects of interferon. Interferons are activated by viral infection and the autocrine loop formed by vIL-6 appears to block antiviral effects induced by KSHV infection itself. A consequence of vIL-6 hypersecretion may include neighboring cell proliferation as is seen in multicentric Castleman's disease (Parravicini *et al.*, 1997). As an interesting aside, this demonstrates that a virus can sense and modify its environment, a property referred to as irritability, that is part of the fundamental definitions for living organisms.

Inhibition of cell cycle progression: K-BZIP/RAP

The proteins thus far described for KSHV act on cell cycle checkpoints to prevent cell cycle arrest. KSHV k-BZIP (also known as replication associated protein, RAP) encoded by ORF K8, paradoxically has opposing effects on cell cycle regulation. K-BZIP is an early spliced gene (corresponding by weak sequence homology to the EBV transactivator Zta or ZEBRA) possessing a basic-leucine zipper (bZIP) (Gruffat *et al.*, 1999; Lin *et al.*, 1999; Seaman *et al.*, 1999). This protein interacts with p53 and may sequester p53 (together with the other major lytic transactivator RTA (Gwack *et al.*, 2001b)) to promyelocytic leukemia (PML) bodies, presumably as a means of delaying p53-dependent apoptosis during the early phases of lytic reactivation (Park *et al.*, 2000). Despite this, K-bZIP causes cell cycle arrest through induction of the CDKI p21, a downstream target of p53, and CCAAT/enhancer binding protein-α (C/EBP-α) resulting in G1 arrest during lytic replication (Wang *et al.*, 2003a,b; Wu *et al.*, 2003). K-bZIP is phosphorylated by CDKs (Polson *et al.*, 2001) and also directly interacts with cyclin A/CDK2 complexes contributing to G1 arrest during early lytic replication (Izumiya *et al.*, 2003).

The reasons for this effect remain speculative. Virus-induced cell cycle arrest may seem counterintuitive – particularly in view of the limited resource hypothesis for oncogene teleology developed from analyses of small DNA

Table 30.3. Major KSHV proteins targeting apoptotic control machinery

	Protein	Gene	Target(s)
Latency	LANA1	ORF73	p53
	LANA2 (vIRF-3)	ORF K10.5	p53
	vFLIP	ORF K13	Fas, NF-κB
Lytic or induced	RTA	ORF50	p53
	K-bZIP	ORF K8	p53/p300/CBP
	vIRF-1	ORF K9	p53/p300/CBP/ATM
	vBCL-2	ORF 16	BAX?
	VIAP	ORF K7	BAX, BH-3 proteins?

tumor viruses (Braithwaite and Russell, 2001; Russell *et al.*, 2004), since S phase entry is thought to be required for viral DNA replication. Entry into a "senescent" phenotype, however, might have protective effects in preventing premature apoptosis during lytic replication. It is also unclear if expression of the lytic phase KSHV DNA synthesis enzymes whose cellular counterparts are under E2F-control (e.g., ribonucleotide reductase, thymidylate synthase, dihydrofolate reductase and thymidine kinase) can generate a quasi-S phase state in the face of K-BZIP induced cell cycle arrest. Conceivably, this may allow viral DNA replication during stasis in cellular DNA replication. K-BZIP acts during lytic replication whereas LANA1 and vCYC act during latency (possibly during lytic replication as well) indicating that the different phases of the viral life cycle require different cell cycle manipulations.

KSHV inhibition of apoptosis

Interference with cell cycle checkpoints activates cellular apoptotic pathways through p14ARF and other less well-defined mechanisms, presumably as a means to prevent tumor cell formation (Sherr, 1998, 2004). It is therefore not surprising that KSHV encodes antiapoptotic factors that mitigate this response. What is surprising, however, is the number, range and kind of anti-apoptotic factors encoded by KSHV (Table 30.3, for review see Lagunoff and Carroll, 2003). KSHV also activates survival factors, such as NF-κB, a trait shared with EBV and other B-lymphotrophic viruses.

Apoptotic signaling is divided into intrinsic and extrinsic pathways integrated with each other through the activity of the transcription factor p53 (Fig. 30.4). Extrinsic pathways are activated through apoptotic signaling receptors, such as Fas/CD95 and the tumor necrosis factor receptor (TNFR), whereas intrinsic pathways are activated in response to cellular stress and DNA damage (Danial and Korsmeyer, 2004).

Extrinsic apoptotic signaling is principally an immune response activated through death-inducing receptors by natural killer (NK) cells and cytotoxic lymphocytes (CTL). Receptors of the TNF-Fas receptor family can have either opposing proapoptotic or antiapoptotic signaling responses depending on the mechanism of activation and the cellular context of receptor activation. Extrinsic apoptotic signaling activates caspase cascade signaling which in turn results in mitochondrial release of apoptosis mediators including cytochrome C. p53 has been implicated in increasing apoptotic receptor transcription and priming other components of these pathways (Sheard *et al.*, 2003).

Intrinsic apoptotic signaling directly activates p53 through a series of kinase cascades, ultimately resulting in mitochondrial apoptosis. Although this characterization of this response is rapidly evolving, current evidence suggest that sensors of DNA damage activate signaling cascades that ultimately result in cell cycle arrest and repair or, failing this, apoptosis. Responses may differ between types of DNA damage (e.g., mismatch damage vs. single and double-strand breaks) that activate different repair responses including nucleotide-excision repair, base-excision repair, or homologous and non-homologous recombination (for review see Wood *et al.*, 2001).

One of the initial responses to DNA damage recognition is binding and activation of the MRE11- Rad51-NBS (MRN) complex, which sequentially activates Chk2 and ATM phosphorylation and subsequent phosphorylation of p53 (Banin *et al.*, 1998; Lee and Paull, 2004). The importance of this pathway to viral replication is hinted at by viruses encoding proteins that target these early responses (Weitzman *et al.*, 2004). KSHV vIRF1, for example, binds to and inactivates ATM downstream signaling to p53 (Shin *et al.*, 2006). p53 phosphorylation causes conformational changes that promote p53 binding to specific DNA regulatory elements to initiate transcription of pro-apoptotic protein genes, such as PUMA and NOXA-1, BH3-only members (see below) of the BCL-2 factor family, which act at the mitochondria to initiate mitochondrial apoptotic responses (Oda *et al.*, 2000; Yu *et al.*, 2001). These BH3-only containing BCL-2 factors bind and sequester other anti-apoptotic BCL-2 members, including BCL-2 itself and BCL-x_L (Cheng *et al.*, 2001; Schuler and Green, 2001).

Disarming the guardian: p53 inhibition

p53 has been called the "guardian of the genome" for its critical role in integrating apoptotic and cell cycle arrest signaling, most notably in response to DNA-damaging

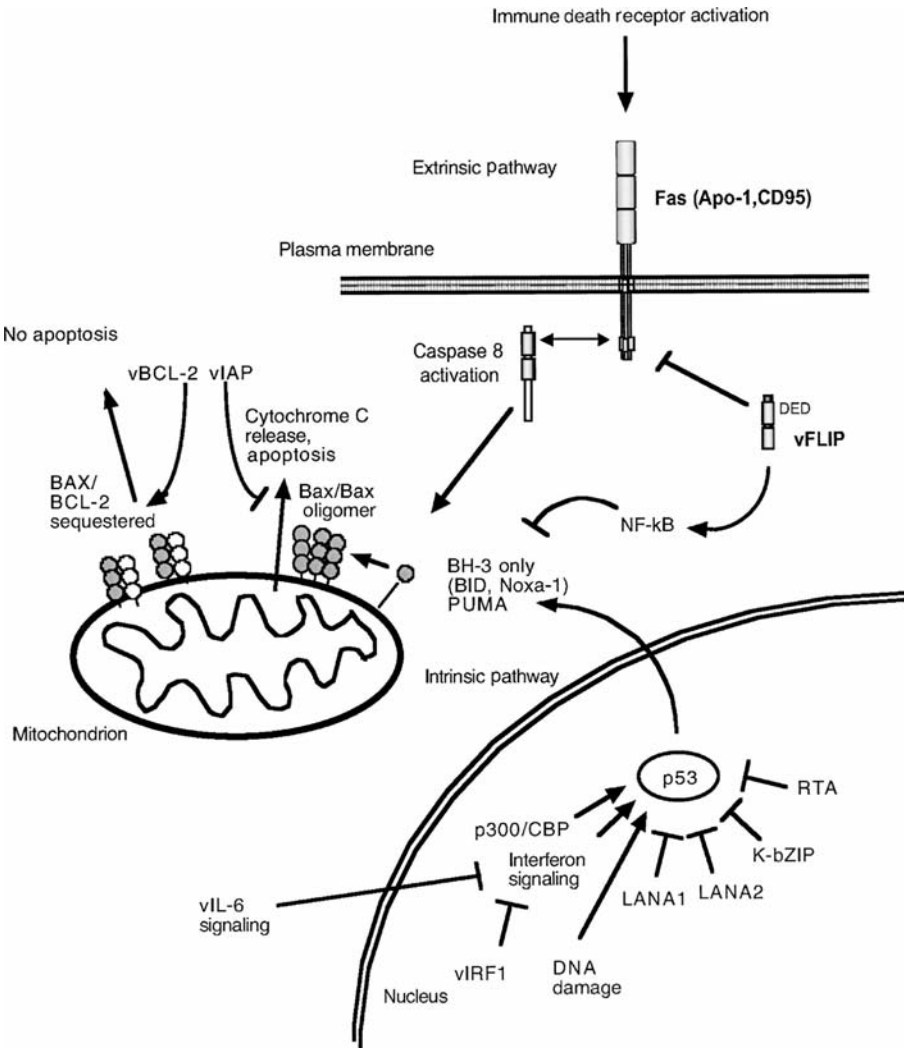

Fig. 30.4. KSHV inhibits both intrinsic and extrinsic apoptotic pathways at multiple points. Extrinsic signaling shown here is activated by cellular immune signaling (NK cell, cytotoxic lymphocyte responses) though activation of the Fas receptor. vFLIP directly inhibits this response through a dominant-negative binding to death effector domains (DED) in the death-inducing signaling complex (DISC) and also activate NF-kB through an alternative pathway which may inhibit B-cell apoptosis at the mitochondria and endoplasmic reticulum. Both vIAP and vBCL-2 act at the mitochondria to stabilize the mitochondrial membrane and inhibit the activating effects of BH3-only pro-apoptotic molecules. The central transcriptional integrator of apoptotic signaling, p53, plays a key role in mediating intrinsic apoptotic effects in response to DNA damage or interferon signaling. KSHV proteins including LANA1, LANA2, K-bZIP and RTA inhibit p53-induced apoptotic signaling either through direct binding or through inhibition of the p300/CBP coactivator used in p53 transcriptional signaling. Delaying apoptosis during lytic viral replication allows efficient production of infectious virions. Apoptotic signaling is also inhibited during latency. The reasons for viral targeting of the cell cycle and apoptotic pathways during latency remain poorly understood.

agents (Lane, 1992). p53 prevents germline transmission of mutations, since DNA damage will either cause p53-dependent arrest allowing repair prior to transmission to daughter cells or, if the damage is irreparable, commits the cell to p53-dependent apoptosis. The molecular decision-making process between apoptosis or cell cycle arrest remains unclear, although p21 has a key role in determining whether p53-activation results in arrest or cell death (Gorospe *et al.*, 1997).

For somatic cells, the roles for p53 are less clear. As a tumor suppressor, p53 may prevent cell transformation resulting from DNA damage. More recently, evidence has accumulated that p53 also acts to prevent viral nucleic acid replication (Moore & Chang, 1998; Takaoka *et al.*, 2003).

Replicating extrachromosomal DNA may activate ATM-p53 signaling, thus serving as an intracellular innate immune response. It is now widely accepted that cellular responses limiting viral replication include apoptosis (Benedict et al., 2002; Meinl *et al.*, 1998), although programmed cell death is not universal for all viral infections and some viruses may actually capitalize on cell dissolution during apoptosis to enhance replication (Teodoro and Branton, 1997).

As a critical integrator of cell cycle regulation and apoptotic signaling, p53 is a common target for tumor viruses that have convergently evolved very different mechanisms to inhibit this major regulatory protein (for review, see Lagunoff and Carroll, 2003). KSHV is no exception to this, and it directly targets p53 during both latency and during lytic replication. Unlike many malignancies, apoptosis in not prominent in KSHV-related disorders, p53 is expressed in tumor cells and p53 mutations do not appear to be common in KSHV-induced tumors (Katano *et al.*, 2001b).

LANA-1

To balance the cell cycle dysregulation effect of vCYC and LANA1 during latency, KSHV must also inhibit p53-mediated apoptotic signaling for infected cell survival. This is most clearly seen in unopposed vCYC transfection assays which induce apoptosis, and vCYC transgenic mice which have a dramatically increased rate of lymphomagenesis in a p53-null background. LANA1 encoded by ORF73 was the first p53 inhibitor discovered in KSHV (Friborg *et al.*, 1999), in which the carboxyl terminus of the protein was shown to interact with p53 and inhibit its transcriptional activator function. This work has since been extended to other rhadinoviruses such as herpesvirus saimiri whose ORF73 protein – while structurally different from the KSHV LANA1 protein – also inhibits p53 activity (Borah *et al.*, 2004).

LANA2/vIRF3

While LANA1 is expressed in all KSHV-infected cells, LANA2 (also known as vIRF3) encoded by the spliced gene ORF 10.5, is expressed exclusively in KSHV-infected hematopoeitic cells. LANA2 shares with LANA1 p53-inhibitory functions although evidence for direct interaction with p53 is less certain due to the inherent "stickiness" of the protein (Rivas *et al.*, 2001). Transient expression of LANA2 abrogates apoptosis caused by activation of p53 signaling or by direct overexpression of p53 protein. Paradoxically, LANA2 simultaneously activates interferon transcription pathways (Lubyova *et al.*, 2004)and inhibits NF-kB signaling pathways (Seo *et al.*, 2004) – two signaling events that would be expected to induce lymphocyte apoptosis. The role, if any, of this molecule in PEL survival and transformation remains largely unknown.

RTA, K-bZIP and vIRF1

KSHV p53-inhibitory proteins active during lytic replication include the lytic transactivator proteins RTA (encoded ORF50) and K-bZIP (encoded by K8 and describe above), and the interferon regulatory factor homologue vIRF1 encoded by ORF K9. These three proteins appear to inhibit p53 transcriptional activity (Gwack *et al.*, 2001b; Park *et al.*, 2000; Nakamura *et al.*, 2001) principally by binding to the p300/CBP family of histone acetyltransferases (HAT) that serve as transcriptional coadaptors for p53 (Gwack *et al.*, 2001a; Hwang *et al.*, 2001; Li *et al.*, 2000; Lin *et al.*, 2001). Direct binding by vIRF1 and K-bZIP to p53 may also occur. HAT activity is required to not only acetylate p53 itself, but also histones to prepare the operon for p53-directed transcription.

By sequestering HATs from the transcriptional complex, these KSHV proteins inhibit transcription not only at p53-regulated promoters but also (in the case of vIRF-1) interferon-regulated promoters (Gao *et al.*, 1997; Lin *et al.*, 2001; Zimring *et al.*, 1998). K-bZIP may also directly sequester p53 to promyelocytic leukemia protein (PML) bodies rendering it inactive for participation in apoptotic signaling (Katano *et al.*, 2001a). The use of CBP/p300 coadaptors is widespread in growth control transcriptional responses, and K-bZIP's ability to sequester CBP/p300 has also been shown to inhibit SMAD3 transcriptional response in the TGF-beta signaling cascade (Tomita *et al.*, 2004).

Beyond p300/CBP sequestration, vIRF1 also activates p53 degradation, possibly by enhancing the E4 ubiquitin-ligase activity of p300/CBP (Shin *et al.*, 2006). vIRF1 has the unusual property of binding to and inhibiting ATM, as well. Thus, vIRF1 has a multiple roles in inhibiting ATM-p53 activation during viral infection.

Induction of p53-mediated apoptosis during lytic viral replication is thus postponed by the activity of several proteins expressed during the earliest phases of viral replication. This presumably forestalls early cell death to optimize virion production. Since cells undergoing full viral replication eventually undergo apoptosis, it is clear that, in the struggle between the virus and the cell, the cell eventually wins–but presumably not before the virus is able to generate infectious progeny. The actual apoptotic triggers initiated by viral replication remain unknown. One possibility is that viral DNA breaks, as a consequence of massive but inefficient viral DNA replication, initiate ATM-p53 signaling. Other possibilities include endoplasmic reticulum stress due to hijacking of cellular protein synthesis machinery or the degradation of cellular mRNA with concomitant

transcriptional inhibition by the KSHV ORF 37 protein (Glaunsinger and Ganem, 2004).

Inhibition of extrinsic apoptotic signaling

vFLIP

KSHV down-regulation of MHC I during both lytic (Coscoy and Ganem, 2000) and latent (Tomescu *et al.*, 2003) viral replication leaves the infected cell open to NK cell attack (see Chapter 31). Intercellular killing is mediated by released membrane toxins (granzyme) and activation of Fas signaling. KSHV vFLIP encoded by ORF K13 potentially abrogates Fas-mediated apoptosis through two dominent-negative death-effector domains (DED) that block the formation of the death-induced signaling complex (DISC) (reviewed in Krueger *et al.*, 2001).

vFLIP encoded by ORF K13 has generated considerable interest as an antiapoptic protein since it is expressed during latency through an IRES in the ORF 72 (vCYC) gene on LT1 and LT2 transcripts (Bieleski *et al.*, 2004; Bieleski and Talbot, 2001; Grundhoff and Ganem, 2001; Low *et al.*, 2001). While its caspase-inhibition functions have a clear benefit in preventing NK immune killing of an infected cell, the ability of this protein to activate NF-kB may have even greater importance in maintaining the infected tumor cell (Chaudhary *et al.*, 1999). NF-kB is constitutively activated in PEL cells through IkB inhibitor phosphorylation due to vFLIP signaling (Field *et al.*, 2003; Matta and Chaudhary, 2004) and this activity may inhibit BH3-only molecule-induced apoptosis (Fig. 30.4). Cesarman and colleagues have demonstrated the importance of this to PEL cell survival using specific NF-kB inhibitors which rapidly induce PEL cell apoptosis (Guasparri *et al.*, 2004; Keller *et al.*, 2000). Differentiating the effects of vFLIP on caspase inhibition from NF-kB activation suggests that the latter is critical for PEL cell survival in tissue culture. vFLIP activates NF-kB through direct interactions with the Ikappa B kinase (IKK) complex which targets inhibition of the NF-kB inhibitor, Ik-B (Liu *et al.*, 2002). Evidence suggests this specifically activates an alternative NF-kB pathway that favors processing of the p52 subunit of NF-kB (Matta and Chaudhary, 2004). Latent expression of vFLIP makes this protein an attractive candidate for contributing to human tumors. Expression of vFLIP enhances tumorigenicity of mouse B lymphoma cells in immunocompetant mice strains (Djerbi *et al.*, 1999), suggesting a role in human KSHV-induced tumors.

The mitochondrial anti-apoptotic proteins: vBCL-2, vIAP

Both intrinsic and extrinsic apoptotic signaling ultimately merge at mitochondria to initiate membrane depolarization, release of cytochrome c and formation of the apoptosomal proteins required for chromatin condensation and endonucleolytic cleavage, volume contraction and the breakdown and blebbing of membrane structures that result in apoptotic cell death (Danial and Korsmeyer, 2004).

As previously indicated, critical components of this process are the BCL-2 family of proteins (BCL-2 referring to the second B-cell lymphoma related rearrangement protein found (Bakhshi *et al.*, 1985) with the cyclin D1 being "BCL-1"). BCL-2 members have up to four conserved BCL-2 homology (BH) domains and dimerize with each other in the mitochondrial membrane. BH1, BH2 and BH3 domains on antiapoptotic members form a hydrophobic pocket which sequesters proapoptotic BH3-only containing members, such as BID, of the BCL-2 family. Some proapoptotic BCL-2 family members, including BAX and BAK, possess all three BH domains but may have specific activation at the BH3 domain which initiates their proapoptotic activity (Danial and Korsmeyer, 2004).

KSHV and other DNA viruses (Cuconati and White, 2002) encode homologues to the cellular BCL-2 antiapoptic protein (Sarid *et al.*, 1997). vBCL2 encoded by ORF16 was the first KSHV protein to be investigated for its apoptosis-inhibitory properties (Cheng *et al.*, 1997; Sarid *et al.*, 1997) and possesses BH1 and BH2 domains. Solution structure studies suggest structural similarities to BH3 and BH4 domains being present (Huang *et al.*, 2002) allowing the KSHV protein to tightly bind pro-apoptotic Bak and Bax peptides, consistent with two-hybrid heterodimerization studies (Sarid *et al.*, 1997). Whereas cellular BCL-2 can be cleaved by caspase proteolysis and converted to a pro-apoptotic version, KSHV vBCL-2 lacks this cleavage site and escapes cellular regulation (Bellows *et al.*, 2000). Also, as previously indicated, vBCL-2 may serve a specific role in KSHV infected cells since it escapes inactivation by the KSHV vCYC protein which can occur for the cellular BCL-2 (Ojala *et al.*, 2000).

Recent studies also suggest an novel role for viral BCL-2 members in preventing cell death. In addition to apoptosis, autophagy – programmed lysosomal degradation of cytosolic components – plays a critical role in inhibiting intracellular pathogens and initiating CD4+ antigen presentation (Schmid *et al.*, 2006). Liang and colleagues demonstrated that the murine γHV-68 vBCL-2 binds to the autophagy signaling complex composed of UVRAG and Beclin-1 (Liang *et al.*, 2006). vBCL-2 inhibition of autophagy is an attractive mechanism for gammaherpesviruses to escape this innate immune signaling pathway. Thus, vBCL-2 may help KSHV and related viruses escape both apoptotic and autophagic surveillance systems.

vBCL-2 is expressed as an early gene during lytic replication (Sarid *et al.*, 1997; Sun *et al.*, 1999), and presumably delays apoptosis to allow optimal virion production (Cuconati and White, 2002). Immunostaining of KS tissues shows vBCL-2 production in a minority of infected spindle cells of advanced nodular lesions also consistent with a role primarily in delaying lytic apoptosis in vivo (Widmer *et al.*, 2002).

Another KSHV molecule acting as an antiapoptotic factor at the mitochondrial member is the recently investigated viral inhibitor of apoptosis protein (vIAP) encoded by ORF K7 (Feng *et al.*, 2002; Wang *et al.*, 2002). KSHV vIAP possesses a BH2 domain and localizes to mitochondrial membranes where it stabilizes mitochondrial membranes from apoptotic Ca+2 depolarization induced by a variety of agents (Feng *et al.*, 2002). While a BCL-2-like interaction may account for some of the antiapoptotic properties of this protein, vIAP interacts with ubiquillin (also known as PLIC1) which regulates the proteosomal machinery. K7 binding to ubiquillin may enhance polyubiquitin-mediated proteolysis of key apoptotic signaling molecules including IK-B and p53 (Feng *et al.*, 2004). vIAP is expressed during lytic replication, but also can be seen immediately after infection with KSHV suggesting a critical role for this protein in preparing the cell for successful viral invasion (Krishnan *et al.*, 2004).

Conclusions

Further, recent studies suggest that many of these "lytic cycle" proteins are actually activated during latency by transcriptional signaling such as notch. Thus, there is a large array of KSHV nonstructural proteins that may contribute cell transformation, and by extension, to KSHV-related tumorigenesis.

In contrast to the small DNA tumor viruses that have only one, or a few, likely viral oncoproteins, KSHV possesses startlingly large number of nonstructural proteins targeting cellular control pathways that regulate cellular proliferation. Multiple KSHV proteins inhibit cellular controls of the cell cycle. Both LANA1 and vCYC directly target negative cell cycle regulators and bypass normal cellular feedback controls that limit cell proliferation. Similarly, KSHV inhibits intrinsic and extrinsic apoptotic signaling at multiple levels using many different proteins, that include direct targeting of p53 as well as targeting both upstream and downstream signaling pathways to p53.

While it is likely that only a few KSHV proteins principally contribute to oncogenesis, it remains to be determined which proteins this are. Latency-expressed proteins are the leading candidates but recent studies reveal that KSHV genes traditionally thought of as being lytic cycle genes can be induced during viral latency. In addition, paracrine contributions to neoplasia have long been thought to be important for KS tumors adding an additional layer of complexity to KSHV-induced cell transformation. One likely reason for the large number of KSHV proteins targeting cellular control machinery is that they act in different tissues that are infected with the virus. This is most obvious example for this is LANA2 which is not appreciably expressed in KS endothelial cells. Similarly, vIRF1 and K1 may have widespread expression in KS tumors that is not found in cultured PEL cells in the laboratory. Although KSHV appears to encode many more 'oncoproteins' than other viruses, this may be more apparent than real. KSHV molecular piracy makes identification of regulatory proteins relatively easy; it is possible that additional, functionally-similar proteins will be found in the other herpesviruses as well.

A more salient question is "Why does KSHV dysregulate the cell cycle and control apoptotic signaling?" One explanation is that during lytic replication, KSHV needs to generate a cell environment that can replicate thousands of copies of viral DNA. This view assumes a passive role for the cell during virus replication. It is becoming increasingly clear, however, that lytic virus replication activates host cell defenses that attempt to shutdown the cell cycle or to initiate apoptotic cell death. While the tumor suppressor feedback control machinery is traditionally thought to be a means of preventing spontaneous tumor cell generation, it works extremely well to also limit virus replication (Moore & Chang, 2003). Evidence for the interplay between innate immune signaling and tumor suppressor signaling (Takaoka *et al.*, 2002) makes it increasingly likely that tumor suppressor pathways have evolved for the equally important role of controlling viral replication (Moore & Chang 1998). Seen from this light, KSHV 'oncoproteins' may actually be innate immunity evasion proteins that allow the virus to escape from hostile cellular responses to virus infection.

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Human gammaherpesvirus immune evasion strategies

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Introduction

The human γ-HVs are able to establish a lifelong, persistent infection that is largely clinically inapparent within the immunocompetent host. However, when these viruses are not kept in check, a variety of lymphoproliferative and neoplastic disorders result that will be detailed elsewhere within this volume. In brief, for HHV-8, also known as Kaposi's sarcoma-associated herpesvirus (KSHV), these neoplasias include Kaposi's sarcoma (KS), multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL). HHV-4, or Epstein–Barr virus (EBV), has been etiologically associated with infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin's disease, hemophagocytic lymphohistiocytosis syndrome and some gastric cancers. Through coevolution with their hosts, these viruses have acquired a number of genes that act to set a fine balance between the uncontrolled, virally driven cellular proliferation seen in the immunocompromised host and complete elimination of infected cells by the immune responses. Several of these gene products cause selective suppression of normal immune system functioning and allow for an apathogenic, persistent infection.

Immune system overview

The immune system provides multiple mechanisms of protection from invading pathogens, whether viral, bacterial or parasitic. These immune responses include both broad spectrum, innate responses and highly specific, adaptive responses. Mechanisms of the innate response include the production of viral replication blocking interferons, opsonization and lysis by the complement cascade and natural antibodies, apoptosis, as well as clearance of infection by natural killer (NK) cells, macrophages, neutrophils and T-cells. The adaptive response mechanisms include the $CD4^+$ T-helper cell directed production of specific, high avidity neutralizing antibodies by the B-cells and elimination of infected cells by antigen-specific cytotoxic T-lymphocytes (CTL). A complex network of protein–protein interactions, providing numerous targets for viral intervention and deregulation, governs all of these processes.

Evasion of innate host immunity

By definition, the innate immune responses are the host's first line of defense against viral infection. These defenses can be roughly broken down into complement-mediated responses (both antibody-dependent and -independent), cytokine responses, apoptotic responses and cell-mediated responses. These responses are broad, able to target multiple pathogens, but by no means non-specific. The innate immune response utilizes a large number of germ line-encoded receptors capable of sensing moieties that are common to many pathogens. Other members of the innate immune system, such as the natural killer (NK) cells, have evolved mechanisms for determining the "health" of a cell by examining the cell for changes in the repertoire of surface molecules. This complexity built in to the innate immune responses excludes the possibility of non-specific targeting of host cells, but also provides a number of regulatory checkpoints which invading pathogens can usurp. Both of the known human γ-HVs have devised a number of strategies for thwarting the efforts of the innate immune system to clear them from the body and for deregulating the cross-talk between the innate and adaptive responses.

Complement deregulation

The complement response is mediated by a series of heat-labile plasma proteins, each given a number designation, whose cleavage and activation from an inactive circulating zymogen is controlled by a host of regulatory proteins. It is initiated through one of three pathways: the classical, alternative or mannan-binding-lectin-associated serine protease (MASP) pathways (for review see Medzhitov and Janeway, 2000). Triggering of the complement response results in activation and cleavage of the first zymogen to its active form, which then in turn cleaves and activates the next zymogen in the cascade. After cleavage, the resulting zymogen products are given lettered subscripts. For example, the cleavage of the C4 zymogen by active C1 results in production of C4a and C4b products (Fig. 31.1). The complement system protects against infection by both bacteria and viruses in three different ways. First, several of the complement proteins, when activated, can covalently bind to pathogens in a process called opsonization. Complement receptor-bearing phagocytes can then internalize and clear the infecting organism. This opsonization also contributes to the activation of the adaptive, humoral response. Second, multiple complement protein cleavage products act as anaphylotoxins, recruiting and activating circulating phagocytes. Third, several activated complement proteins can form a large multimeric structure called the membrane attack complex (MAC). This protein complex is capable of creating a pore in lipid membranes resulting in the lysis of cells, enveloped virus or bacteria onto which it has been deposited. While each pathway is initiated by a different triggering event, they converge at the production of the multi-component C3 convertase and ultimately, each result to different degrees in opsonization, anaphylotoxin production and MAC formation. Regulation of the complement cascade is complex, involving proteins that mediate the degradation of complement components into inactive fragments, as well as other inhibitory proteins capable of irreversible binding to and inactivation of complement proteins. The γ-HVs have taken advantage of this complexity by encoding viral genes that interfere with this regulation which are outlined in Table 31.1.

KSHV encodes a homologue of the human complement control protein CD46 (Neipel *et al.*, 1997a,b). Like its human homologue, the ORF4 gene product, termed KCP or Kaposica, contains four short consensus (SCR) or sushi domains. These domains are characteristic of the cellular regulators of complement activation (RCA) (Klickstein *et al.*, 1987; Law, 1988). The SCR are typically 60–70 amino acids in length and contain four conserved cysteine residues, which are disulfide-linked. KCP/Kaposica is encoded from a 1650 nucleotide long open reading frame and work by Spiller *et al.* (2003) has demonstrated that it is expressed as three alternatively spliced constructs, an unspliced 550 residue form and two singly spliced forms of 425 residues and 347 residues (depicted in Fig. 31.2). All three forms retain the putative membrane-spanning region. The unspliced product (ORF4-F) has 14 N-X-S/T, consensus N-linked glycosylation sites. However, the NetNGlyc neural network N-linked glycosylation prediction program (http://www.cbs.dtu.dk/services/NetNGlyc) (Gupta *et al.* 2002), indicates that only 10 of these have a significant probability of being glycosylated. The other two forms, designated ORF4-M and ORF4-S, have five and four probable sites, respectively. Additionally, ORF4-F has potential for modification by O-linked glycosylation in a serine/threonine-rich region just upstream of the predicted transmembrane domain, while the other forms lack this region. Examination of the lysates of TPA-treated PEL cells showed three anti-ORF4 antibody reactive bands at 175 kD, 82 kD and 62 kD (Spiller *et al.*, 2003). Examination of culture supernatants demonstrated the presence of only the two more slowly migrating forms. At this time, the contribution of glycosylation or additional post-translational modifications to the higher than predicted molecular weights of these products or differences in the functioning of each product has not been clarified. Since all of the proposed products maintain a trans-membrane domain, the mechanism of secretion also needs further investigation.

KCP/Kaposica strongly enhances the decay of the classical C3 convertase (C4b2a) but poorly promotes decay of the alternative pathway C3 convertase (C3bBbP) compared with the host complement control proteins (Mullick *et al.*, 2003; Spiller *et al.*, 2003). It acts as a co-factor to aid Factor I (fI), a major cellular complement control protein, in its degradation of both C4b and C3b (Fig. 31.1). Unlike cellular fI co-factors, however, KCP/Kaposica is able to drive production of the C3d complement protein as a final product of fI-mediated cleavage of iC3b (Spiller *et al.*, 2003). Production of this molecule is usually driven by a cellular protease in a non-fi-dependent manner. In addition to accelerating decay of the C3 convertase, thus preventing the action of complement, this production of C3d by KCP/Kaposica probably plays an additional role in KSHV biology. The C3d molecule is capable of binding to complement receptor 2 (CR2, CD21), which complexes with CD19 and CD81, resulting in a dramatic increase in B cell responsiveness to B cell receptor stimulation (Dempsey *et al.*, 1996). So, the actions of KCP/Kaposica likely have some effects on B cell production of antibodies in response to viral antigens. Whether this directly alters anti-viral responses or aids the

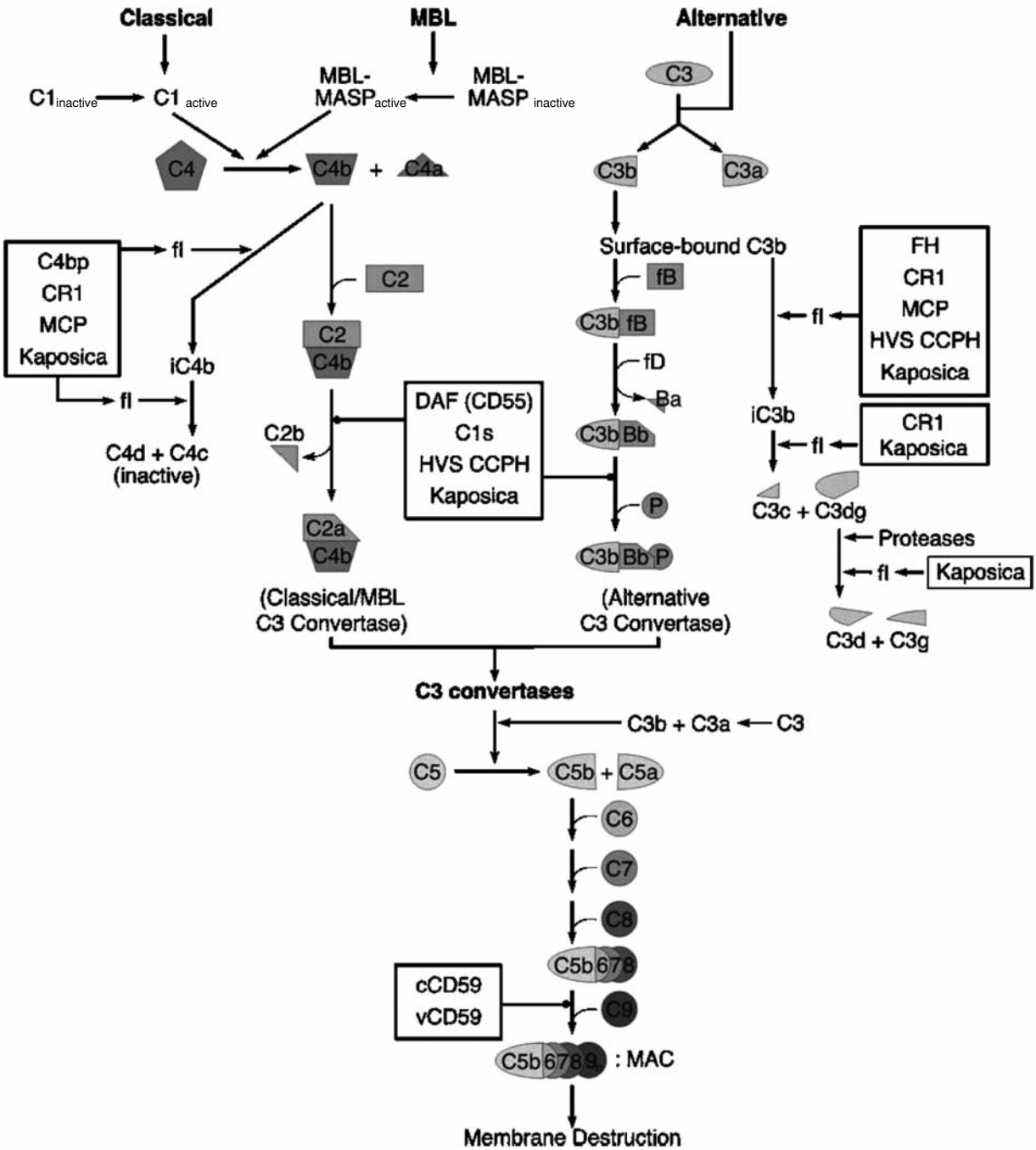


Fig. 31.1. Outline of the Complement cascade with interfering herpesvirus products noted. The complement cascade is a complex series of zymogens and regulating proteolytic enzymes. This high-level of complexity gives multiple opportunities for intervention by the herpesviruses. Additional details are given in the text.

Table 31.1. Viral complement regulators. Complement forms an important facet of the innate immune response. Not only does it play a role in direct defense, through lysis of infected cells or enveloped viruses, but it also helps to co-ordinate later adaptive responses. Both human γ-herpesviruses encode gene products with the potential to alter the complement response. The open reading frame, product and potential function are listed for each, with additional details in the text

	Gene	Product	Function
HHV-8	ORF4	KCP/Kaposica	Homologue of human CD46, accelerates the decay of the C3 convertases and drives production of C3d, which binds complement receptor 2 (CR2)
EBV	BLLF1a/b	gp350/220	Binds to CR2, might interfere with complement mediated B cell stimulation or aid in viral dissemination

ORF4-F
MAFLRQTLWILWTFTMVIGQDNEKCSQKTLIGYRLKMSRDGDIAVGETVELRCRSGYTTY ARNITA
TCLQGGTWSEPTATCNKKSCPNPGEIQNGKVIFHGGQDALKYGANISYVCNEGYFLVGREYVRYCM
IGASGQMAWSSSPPFCEKEKCHRPKIKNGDFKPDKDYYEYNDAVHFECNEGYTLVGPHSIACAVNNT
WTSNMPTCELAGCKFPSVTHGYPIQGFSLTYKHKQSVTFACNDGFVLRGSPTITCNVTEWDPPLPKC
VLEDIDDPNNSNPGRLHPTPNEKPNGNVFQRSNYTEPPTKPEDTHTAATCDTNCEQPPKILPTSEGFNETT
TSNTITKQLEDEKTTSQPNTHITSALTSMKAKGNFTNKTNNSTDLHIASTPTSQDDATPSIPSVQTPNYNTNA
PTRTLTSLHIEEGPSNSTTSEKATSSTLSHNSHKNDTGGIYTTLNKTTQLPSTNKPTNSQAKSSTKPRVETHN
KTTSNPAISLT**DSADVPQRPREPTLPPIFRPPASKNRYLEKQLVIGLLTAVALTCGLITLFHYLF FR**

ORF4-M
MAFLRQTLWILWTFTMVIGQDNEKCSQKTLIGYRLKMSRDGDIAVGETVELRCRSGYTTYARNITA
TCLQGGTWSEPTATCNKKSCPNPGEIQNGKVIFHGGQDALKYGANISYVCNEGYFLVGREYVRYCM
IGASGQMAWSSSPPFCEKEKCHRPKVKNGDFKPDKDYYEYNDAVHFECNEGYTLVGPHSIACAVNN
TWTSNMPTCELTGCKFPSVTHGYPIQGFSLTYKHKQSVTFACNDGFVLRGSPTITCNVTEWDPPLPK
CVLEDIDDPNNSNPGRLHPTPNEKPNGNVFQRSNYTEPPTKPEDTHTAATCDTNCEQPPKILPTSEGFNET
TTSNTITKQLEDEKTTSQPNTHITSALTSMKAK**DSADVPQRPREPTLPPIFRPPASKNRYLEKQLVIGLLT**
AVALTCGLITLFHYLF FR

ORF4-S
MAFLRQTLWILWTFTMVIGQDNEKCSQKTLIGYRLKMSRDGDIAVGETVELRCRSGYTTYARNITA
TCLQGGTWSEPTATCNKKSCPNPGEIQNGKVIFHGGQDALKYGANISYVCNEGYFLVGREYVRYCM
IGASGQMAWSSSPPFCEKEKCHRPKVKNGDFKPDKDYYEYNDAVHFECNEGYTLVGPHSIACAVNN
TWTSNMPTCELTGCKFPSVTHGYPIQGFSLTYKHKQSVTFACNDGFVLRGSPTITCNVTEWDPPLPK
CVLEDIDDPNNSNPGRLHPTPNEKPNDSADVPQRPREPTLPPIFRPPASKNRYLEKQLVIGLLTAVALT
CGLITLFHYLF FR

Fig. 31.2. Products of the Kaposica/KCP open reading frame. The Kaposica/KCP open reading frame has multiple splice forms. Regions in bold are shared by all three isoforms. The underlined region is found in both the M and F forms. Green residues indicate the location of the potential transmembrane domain. Blue residues indicate areas of potential N-linked glycosylation with the first residue colored red if the NetNGlyc neural network prediction program determined that that site was above the predicted probability threshold for glycosylation.

virus in recruiting additional target cells or possibly altering viral entry is still unclear.

While no EBV-encoded proteins to date have been shown to have a direct effect on complement regulation, EBV also targets CR2. The gp350/220 viral envelope protein is capable of binding to and mediating viral entry into $CD21^+$ cells. The binding of gp350/220 to CR2, while not identical to C3d, probably overlaps the complement binding region (Moore *et al.*, 1991; Prota *et al.*, 2002). However, it is currently unclear if binding to this receptor by EBV significantly alters complement activation or responses. Again, it is possible that the virus utilizes this ability to aid in dissemination without effects on complement. It is clear, however, that binding of EBV or the gp350/220 glycoprotein to CR2 has effects on cytokine production and survival for several cell types and will be discussed in later sections.

A number of other viruses have been shown to incorporate host complement regulatory proteins into their

envelopes including human cytomegalovirus and HIV (Saifuddin *et al.*, 1994; Spiller *et al.*, 1996). While no experimental evidence has yet been shown for a similar strategy employed by the human γ-HVs, this is a tantalizing possibility.

Cytokine responses

The cytokines are a large number of mostly soluble proteins, able to bind to a wide variety of cellular receptors expressed both on other immune effectors and non-immune related host cells. Through binding to their receptors they can induce proliferation, differentiation and activation both in the producer cell (autocrine effects) and in other targets (paracrine effects). Included in this large group of proteins are several super-families of proteins including the interleukins, interferons and chemokines. Generally, the interleukins (usually given an IL designation) are proteins that are produced by one leukocyte and act on another, however numerous examples exist that don't fall into this general definition. These proteins act to attract and activate a number of immune effector cells, activate the host acute-phase response and drive the differentiation of cells to result in a polarized immune response. The interferons (IFNs) are an evolutionarily conserved group of proteins that play a crucial role in the innate response to viruses. These proteins are able to mediate signaling through their receptors to induce the expression of a large number of cellular proteins that are able to alter cellular physiology to be less hospitable for viral replication. The chemokines interact with cellular 7-transmembrane, G-protein-coupled receptors (GPCRs) stimulating leukocyte trafficking and development, as well as regulating angiogenesis. Crucial to immune system functioning is their ability to recruit various effector cells to the site of inflammation. The spectrum of chemokines that are produced govern which cells respond to the site of inflammation and have a large influence in directing how the immune system reacts to invading pathogens. The signals that these cytokines transmit to the cells can stimulate the production of additional cytokines and chemokines, providing a complex cross-talk evolved to coordinate an effective immune response.

Two main subpopulations of $CD4^+$ T lymphocytes, termed T helper (Th)1 and Th2 cells, coordinate the type of immune response that is made to an infecting pathogen. Th1 cells predominantly secrete IFN-γ, GM-CSF and TNF-α, but also secrete TNF-β, IL-3 and IL-2. Additionally, they usually express CD40 ligand and/or CD95 ligand on their surface. These types of cells act to activate macrophages, direct B-cells to produce opsonizing antibodies and cause inflammatory cell infiltration of tissues. Thus, the Th1 cells select for cell-mediated immune responses against pathogens. Th2 cells secrete IL-4 and IL-5, but also IL-10, TGF-β, eotaxin and IL-3. They also express CD40 on their surface. These types of cells act to activate antigen-specific B-cells to produce neutralizing antibodies and thus direct the immune effectors toward a humoral response against the invading pathogen. Additionally, the cytokines released by Th2 cells tend to inhibit inflammation. These two populations of T-lymphocytes result from the differentiation of naïve $CD4^+$ T-cells in response to the cytokine milieu, cytokines which have been released from either infected cells or other immune effectors such as NK cells. Shifts in the dominance of Th1 or Th2 immune effectors can dramatically influence the type and importantly, the effectiveness of responses made against invading pathogens.

Interleukin and chemokine responses

KSHV expresses multiple chemokine homologues, homologues of the macrophage inflammatory proteins, vMIP-I, -II and –III, a homologue of the cellular IL-6 protein and a homologue of the cellular Ox2 protein, which is involved in the release of a number of different cytokines. A summary of these gene products is outlined in Table 31.2. The viral MIPs are able to bind to a variety of cellular chemokine receptors, acting as agonist and antagonists (Boshoff *et al.*, 1997; Kledal *et al.*, 1997; Sozzani *et al.*, 1998; Dairaghi *et al.*, 1999; Endres *et al.*, 1999; Stine *et al.*, 2000). The gene products of ORFs K6 and K4 of KSHV, vMIP-I (vCCL1) and v-MIP-II (vCCL2) respectively, share homology with the cellular CC chemokine macrophage inflammatory protein-1 alpha (MIP-1α) (Moore *et al.*, 1996; Neipel *et al.*, 1997a,b). The similar size of the K6 (95 residues) and K4 (94 residues) products and their high degree of sequence identity suggest that they arose through a gene duplication event. The third chemokine homologue, vMIP-III (vBCK, vCCL3) is 114 residues in length, encoded by the K4.1 ORF and has homology with MIP-1β as well as several other members of the cellular CC chemokine family (Neipel *et al.*, 1997a,b; Stine *et al.*, 2000). The target of the vMIPs seems to be the Th2 lineage $CD4^+$ T cells based on the fact that vMIP-I can act to induce chemotaxis of CCR8-bearing cells; vMIP-II, chemotaxis of CCR3-bearing cells; vMIP-III chemotaxis of CCR4 -bearing cells, and all of these receptors are found on Th2 cells (Sallusto *et al.*, 1998) (Fig. 31.3). Each of the vMIPs have been shown to induce the chemotaxis of Th2 cells (Stine *et al.*, 2000). Further, Weber *et al.* demonstrated that vMIP-II is able to block the chemotaxtic effects of RANTES on Th1 cells and monocytes, thus inhibiting their recruitment to sites of vMIP production (Weber *et al.*, 2001) (Fig. 31.3). Nakano *et al.* (2003), in contrast, demonstrated

Table 31.2. Viral chemokine regulators. Recruitment of immune effectors is critical to the generation of an effective immune response. The soluble chemokines play a large role in this recruitment. Both human γ-herpesviruses encode multiple gene products with the potential to alter the chemokine response. The open reading frame, product and potential function are listed for each, with additional details in the text

	Gene	Product	Function
HHV-8	K6	vMIP-I (vCCL1)	Alters chemotaxis, causes Th2 polarization, neo-angiogenesis, VEGF-A elicitation
	K4	vMIP-II (vCCL2)	Alters chemotaxis, causes Th2 polarization, neo-angiogenesis
	K4.1	vMIP-III (vCCL3, vBCK)	Alters chemotaxis, causes Th2 polarization
	K2	vIL-6	Acts as growth factor, stimulates elicitation of VEGF-B, causes Th2 polarization and Th1 inhibition, activates multiple signaling cascades
	Orf13	vFLIP	Binds TRAF2 causing the induction of cIL-6, causes Th2 polarization, acts as a growth factor
	K14	vOX2	Stimulates macrophage/monocytes to release IL-1β, IL-6, MCP, causes Th2 polarization, may have role in viral dissemination
EBV	LMP-1/EBI-3	cIL-12 subunit	Alters IL-12 signaling, Th2 polarization, might alter IL-4 and IFN-γ production, might effect NKT cells
	BCRF-1	vIL-10	Blocks IFN-γ and IL-2 production, inhibits dendritic cell maturation, Th2 polarization

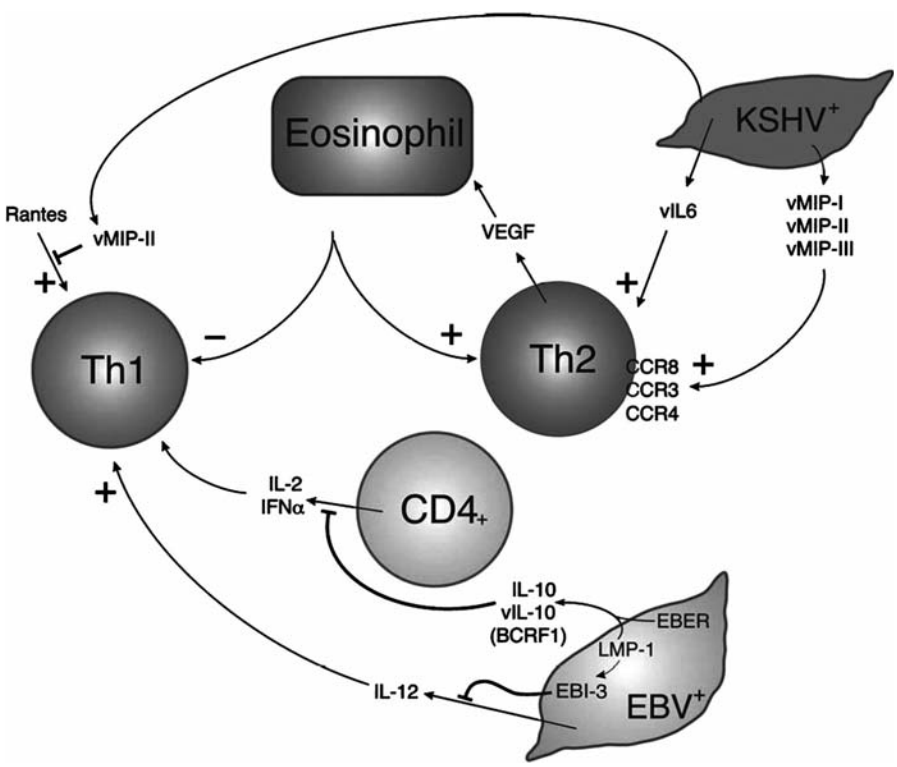


Fig. 31.3. Herpesvirus skewing of the Th1/Th2 balance by multiple gene products. Both KSHV and EBV express multiple gene products that mimic cellular cytokines. These proteins have multiple effects on a number of immune effectors including the $CD4^+$ T helper cells, further detailed in the text. The web of viral and cellular cytokines have a net effect of favoring a Th2, humoral response.

that vMIP-I and –II induced chemotaxis of a monocyte cell line. However, these two groups used different experimental designs and much different target cells, a transformed monocyte cell line versus primary monocytes. The majority of the published data suggests that it is to the advantage of the virus to polarize the immune response to a Th2 pattern, suggesting that cellular adaptive immune responses are more likely to clear the virus from the body.

The vMIP proteins are also likely to have other effects in addition to their role in immune evasion. Both the vMIP-I and –II proteins are highly angiogenic in the chorioallantoic assay (Boshoff *et al.*, 1997). Additionally, vMIP-1 can induce chemotaxis of endothelial cells through its interactions with the CCR8 receptor (Endres *et al.*, 1999). Combined, this suggests that the vMIP proteins might be playing a large part in directing the development of the structure of the vascular KS lesions. Liu *et al.* (2001) have shown that addition of the vMIP-I protein to PEL cell lines resulted in the expression of vascular endothelial growth factor type A (VEGF-A). This VEGF-A can have both paracrine and autocrine effects, influencing PEL cell growth, extravasation and recruitment of other cells such as eosinophils, which can release Th2-type cytokines (Weber *et al.*, 2001; Feistritzer *et al.*, 2003) (Fig. 31.3). Again, pointing to a dual role for the vMIP proteins in directing KS lesion formation as well as altering anti-viral immune responses.

Further, supporting the thesis that KSHV better survives a Th2-biased immune response, the virus produces yet another soluble factor which favors the development of a Th2 response. Viral IL-6, encoded by KSHV ORF K2 is 204 residues in length and has approximately 25% homology with human IL-6 (Neipel *et al.*, 1997a,b; Nicholas *et al.*, 1997). Message for vIL-6 is very rapidly transcribed after infection (within 2 hours) and then is just as quickly downregulated, disappearing by 8 hours post-infection (Krishnan *et al.*, 2004). Cellular IL-6 (cIL-6) performs multiple functions, including inducing the differentiation of B-cells into antibody-secreting plasma cells (Beagley *et al.*, 1989). It promotes Th2 differentiation and simultaneously inhibits Th1 polarization through two independent molecular mechanisms. It also acts as both an autocrine and paracrine growth factor, delivering a signal through the IL-6 receptor, a heterodimer made up of gp80 and gp130 (Kawano *et al.*, 1988; Klein *et al.*, 1989; Kishimoto *et al.*, 1995; Nakashima and Taga, 1998) (Fig. 31.3). This signal in many ways resembles the signal transmitted by INF-α/β interactions with its receptor, proceeding through members of the JAK kinase family to transmit a signal to the cellular STAT3, which can then stimulate transcription of a group of IL-6 responsive genes (Murakami *et al.*, 1993; Narazaki *et al.*, 1994; Stahl *et al.*, 1994; Guschin *et al.*, 1995). Additionally, STAT1 also becomes phosphorylated, dimerizing with STAT3 to bind and transactivate genes containing interferon-inducible GAS sequences (Feldman *et al.* 1994; Lutticken *et al.*, 1994; Wegenka *et al.*, 1994; Guschin *et al.*, 1995). The stimulation of cells with cIL-6 also causes the phosphorylation of STAT5 and the induction of the MAP kinase pathway (Diehl and Rincon, 2002).

The KSHV vIL-6 protein, like cIL-6, has been shown to perform numerous functions. It is capable of activating STAT1, 3 and 5, as well as the MAP kinase signaling cascade (Molden *et al.*, 1997; Osborne *et al.*, 1999; Hideshima *et al.*, 2000). However, unlike cIL-6, only the gp130 subunit of the IL-6 receptor is required, although the gp80 subunit can allow vIL-6 to signal more promiscuously (Molden *et al.*, 1997; Aoki *et al.*, 1999; Wan *et al.*, 1999; Li *et al.*, 2001; Klouche *et al.*, 2002; Li and Nicholas, 2002). This ability to signal through the gp130 subunit alone probably allows vIL-6 to continue to transmit anti-apoptotic and growth stimulatory signals even after down regulation of the p80 protein, a normal cellular response to cIL-6 signaling. KSHV vIL-6 likely plays a critical role in the pathology of the virus. There are greater levels of expression in MCD and PEL samples than KS lesion samples, and one group has demonstrated high level vIL-6 production in KSHV-associated germinotropic lymphoproliferative disorder, a rare, newly described disease (Boshoff *et al.*, 1996; Parravinci *et al.*, 1997; Staskus *et al.*, 1997; Du *et al.*, 2002). Both cIL-6 and vIL-6 transmit a proliferative signal to PEL cell lines, both through induction of VEGF-B and induction of pro-survival pathways (Neipel *et al.*, 1997a,b; Nicholas *et al.*, 1997; Burger *et al.*, 1998; Jones *et al.*, 1999; Hideshima *et al.*, 2000; Liu *et al.*, 2001). Although there is great overlap in the functions of vIL-6, Foussat *et al.* demonstrated a clear need for cIL-6 in PEL cell tumor progression in mice (Foussat *et al.*, 1999). Antibodies against cIL-6 slowed tumor growth even though vIL-6 was still being produced. In addition to a role of vIL-6 in delivering a positive growth signal and protecting from programmed cell death, it probably plays additional roles important to immune effector avoidance. As described above, cIL-6 is critical in driving immunoglobulin production from committed B-cells. The KSHV vIL-6 probably plays a similar role, further polarizing the immune responses, along with the other virally-encoded or –induced cytokines, toward a Th2 response. More recently, Klouche *et al.* demonstrated that unlike cIL-6, vIL-6 can induce the production of pentraxin-3 (PTX-3, TSG14) (Alles *et al.*, 1994; Klouche *et al.*, 2002). This acute-phase protein is capable of binding to apoptotic cells and preventing their recognition by dendritic cells, possibly preventing auto-immunity during the acute phase response in which there is a high amount

of cell death (Rovere *et al.*, 2000). Therefore, it is possible that the production of PTX-3 by vIL-6 helps to reduce recognition of virally-infected cells by APC. However, since PTX-3 only binds to C1q, the initiating protein of the classical complement pathway, or the surface of cells undergoing programmed cell death, the ability of vIL-6 to aid in immune avoidance through this mechanism requires additional experimental examination (Rovere *et al.*, 2000; Mantovani *et al.*, 2003; Nauta *et al.*, 2003). Further underlining a potentially high importance of IL-6 to KSHV persistence or replication is the finding that the vFLIP protein, discussed later in detail, can induce the production of cIL-6 through interactions with TRAF2, which activates the JNK/AP1 pathway and induces IL-6 synthesis (An *et al.*, 2003).

The cellular OX2 protein (CD200) plays a role in co-stimulation of activated T-cells and suppression of monocyte lineage cell responses (Borriello *et al.*, 1997; Gorczynski *et al.*, 1999). It has been shown to provide a co-stimulatory signal for activated T-cells, leading to an increase in IL-4 and TGF-β, but not IL-2 production (Borriello *et al.*, 1997). In contrast OX2 delivers a negative signal to macrophage and monocytes, inhibiting their proliferation (Gorczynski *et al.*, 1998; Gorczynski *et al.*, 1999). Work by Foster-Cuevas *et al.* (2004) has shown that the interaction of OX2 with its receptor (CD200R) on activated macrophages results in a block to TNF-α production. In mice lacking OX2, dramatic increases in the macrophage and monocyte populations in the mesenteric lymph nodes supports the hypothesis that OX2 plays a role in negatively regulating these cell populations (Gorczynski *et al.*, 1999).

KSHV encodes a gene product from ORF K14 with approximately 40% homology with human OX2. This viral protein is 271 residues in length, migrates with an apparent molecular weight of 55kDa and contains five putative N-linked glycosylation sites (Chung *et al.*, 2002). The higher than predicted molecular weight and experiments with N-glycosidases suggest that all of the putative carbohydrate modification sites are used (Chung *et al.*, 2002). The expression of vOX2 protein is increased after TPA-treatment of PEL cell lines. Although this homology is rather low it is still able to bind to CD200R with affinity and kinetics similar to OX2 (Foster-Cuevas *et al.*, 2004). The biological activity of this viral OX2 is controversial. The experimental work of Chung *et al.* (2002) demonstrated that the viral protein, provided as a soluble GST-coupled protein, delivers a stimulatory signal to macrophage/monocyte and dendritic cells causing them to elicit several pro-inflamatory cytokines including interleukin-1β (IL-1β), IL-6, monocyte chemoattractant protein 1 (MCP-1), and TNF-alpha. Further, expression of vOX2 on the surface of a B-cell line could stimulate the production of TNF-α and IL-12 from U937 cells in the presence of IFN-γ. In contrast, Barclay's group found that when vOX2 expressed on the surface of Chinese hamster ovary (CHO) cells was presented to human peripheral monocyte-derived macrophages there was no increase in TNF-α production (Foster-Cuevas *et al.*, 2004). To the contrary, there was an inhibition of TNF-α production, similar to what was seen when cOX2 was delivered in a similar manner. Reductions were also seen in the amounts of MCP-1 and G-CSF. However, the experimental methodologies of these two groups were radically different. It is a distinct possibility that the two groups were measuring the effects of vOX2 on different receptors. Further experimental verification is still required to determine the biological activity of this protein. The exact role of this protein and the advantage it conveys upon this virus also still requires further study. One possibility is that the virus is altering the cytokine response profile to control or misdirect anti-viral immune effector proliferation and recruitment. Increases in IL-6 production would be expected to bias $CD4^+$ Th2 cell generation. However, it is also possible that the virus is utilizing cellular cytokines to induce proliferation/recruitment of additional target cells as well as facilitating cytokine-mediated angiogenic proliferation to aid in viral dissemination. If vOX2 has a negative effect on monocyte stimulation it could be acting within KS lesions to block responses from the local infiltrating macrophages which could recruit other immune effectors.

EBV expresses a variety of genes that influence the cellular cytokine milieu. As mentioned previously, LMP-1 induces the expression of EBI-3 resulting in decrease in IL-12 production and increases in IL-27 secretion (Devergne *et al.*, 1996, 1997). This has effects on IFN-γ and Th1 responses from both monocytes and $CD4^+$ T cells (Nieuwenhuis *et al.*, 2002; Pflanz *et al.*, 2002) (Fig. 31.3). Further, the BCRF1 gene, a homologue of IL-10, also modulates cytokine production. Its expression limits the production of both IL-1 and IL-2 from $CD4^+$ T-cells (de Waal Malefyt *et al.*, 1991; Liu *et al.*, 1997; Zeidler *et al.*, 1997; Hayes *et al.*, 1999). vIL-10 can also alter the responsiveness of dendritic cells to MIP-1α and MIP-1β. Although vIL-10 is expressed during the lytic program, LMP1 and the EBERs have been shown to induce production of cIL-10. Examination of immune responses against epitopes in LMP1 in EBV^+ and EBV^- individuals demonstrated that the majority of responding cells were Th1 cells, which secrete IL-10, suppressing T-cell proliferation and IFN-γ production (Marshall *et al.*, 2003) (Fig. 31.3). In addition to these specific genes that have been mentioned, EBV likely possesses a number of additional mechanisms for altering the host chemokine and cytokine profile. These changes seem to play a large role in driving the pathophysiology of EBV infection.

As is seen for KSHV, EBV seems to alter the cytokine milieu to favor the development of a Th2 response. The IL-12 protein influences naïve CD4 T-cells to differentiate toward a Th1 profile. EBI-3 decreases IL-12 production, thus reducing one pro-Th1 factor. Further cellular IL-10 can inhibit Th1 cell generation. The BCRF1 viral protein likely plays a similar role and additionally, like cellular IL-10, inhibits the Th1 cytokines IL-2 and IFN-γ. Again, further in vivo investigation is required to better understand the correlates of an effective immune response against the γ-HVs.

Interferon responses

The interferons (IFN) are a family of critically important cytokines that act to modulate cell proliferation and play an important role in innate and adaptive immunity. The type I IFN-α/β and IFN-ω are produced by virally infected cells, whereas the type II IFN-γ is produced by innate immune system effectors cells, as well as adaptive response effectors such as Th1 cells and $CD8^+$ cells (Biron *et al.*, 1999). These proteins act by binding a variety of cell surface receptors and transmitting a signal through the Janus protein kinases (JAKs) and signal transducers and activators of transcription (STATs) to induce expression of a variety of interferon response factors (IRFs) (reviewed in Leonard, 2001; Sato *et al.*, 2001; Taniguchi *et al.*, 2001). In turn, these IRFs regulate the transcription of a wide variety of genes containing either IFN-stimulated response elements (ISRE) or γ-interferon activation sequences (GAS) in their promoters. Regulated proteins include multiple protein kinases, the tumor necrosis factor receptor and MHC class I and class II proteins (Pober *et al.*, 1983; Collins *et al.*, 1984; Boehm *et al.*, 1997; Stark *et al.*, 1998). The interferon responsive proteins act to induce an anti-viral state in the expressing cells, targeting multiple steps in the life cycle of the virus. For example, through the action of the IFN-responsive oligoadenylate synthase, RNase L is activated. This endoribonuclease is active against double-stranded RNA (dsRNA), thus targeting those viruses with either dsRNA genomes, such as the *Reoviridae* (Miyamoto *et al.*, 1983), or containing extensive dsRNA structure, such as the *Picornaviridae* (Robberson *et al.*, 1982). Another major IFN-inducible gene is the protein kinase PKR, capable of phosphorylating the eIF-2α initiation factor, inhibiting mRNA translation and blocking the production of viral proteins (Thomis and Samuel, 1992). Yet a third major antiviral interferon response is the induction of the MxA protein. MxA is a dynamin-like GTPase that is able to bind to the nucleocapsids of some viruses altering their intracellular transport (Haller and Kochs, 2002; Kochs *et al.*, 2002). Further, the IFNs are able to stimulate the production of a number of other chemokines and cytokines, influencing the immune response to invading pathogens.

Activation of the IFN response can occur in at least two ways. First, as described above, binding of interferon to its cognate receptor on the surface of the cell transmits a signal that modulates the IRFs. However, this mechanism requires that IFNs already have been synthesized, either by the responding cell or a surrounding cell. Activation can also occur by a much more direct method. The cellular IRF-3 is part of a complex of proteins called the double-stranded RNA-activated transcription factor complex (DRAF1), which also includes p300/CREB binding protein (CBP) (Weaver *et al.*, 1998). DRAF1 is sensitive to the presence of dsRNA and becomes serine/threonine phosphorylated upon viral infection. This results in a nuclear accumulation of DRAF1, where it binds to and activates ISRE sequences (Kumar *et al.*, 2000). This results in the up regulation of a number of "interferon-responsive" genes as well as production of IFN-α and -β, which can then act in both autocrine and paracrine activation of the interferon pathway (Weaver *et al.*, 1998). A schematic of the multiple mechanisms that the herpesviruses use to interfere with the IFN response pathway is given in Fig. 31.4 and detailed in the text below.

KSHV was the first virus identified as carrying an IRF (Moore *et al.*, 1996). The K9 protein, vIRF-1, is a 449 residue product with some limited homology to the cellular IRFs (Moore *et al.*, 1996). It is capable of inhibiting both type I and II interferon signaling and additionally has transforming activity (Gao *et al.*, 1997; Li *et al.*, 1997; Inagi *et al.*, 1999) (Table 31.3). Expression in NIH 3T3 cells allows growth in soft agar and at low serum concentration (Gao *et al.*, 1997). Further, these vIRF-1-expressing NIH 3T3 cells lose contact inhibition and are tumorigenic in nude mice. These activities of vIRF-1 are the result of interactions with multiple cellular proteins including the cellular IRF-1 and IRF-3 transcription factors, however it does not effect IRF-7-mediated transactivation (Lin *et al.*, 2001). Interactions with these cellular IRFs block production of IFN-β and the RANTES chemokine, important in directing the infiltration of a number of leukocytes including NK and effector T-cells (Lin *et al.*, 2001). These interactions also block the ability of IRF-1 and IRF-3 to direct transcription from the ISG and IFNA4 promoters. However, at least for IRF-3, binding by vIRF-1 did not effect dimerization, nuclear translocation and DNA binding activity. Rather, vIRF-1 interacted with the p300/CBP and efficiently inhibited the formation of transcriptionally competent IRF-3-CBP/p300 complexes (Lin *et al.*, 2001). The further implications of the ability of vIRF-1 to interact with p300/CBP will be discussed later. Additionally, the interaction of

Table 31.3. Viral interferon regulators. Both human γ-herpesviruses encode multiple gene products with the potential to alter the IFN response. The open reading frame, product and potential function are listed for each, with additional details in the text

	Gene	Product	Function
HHV-8	K9	vIRF-1	Blocks IFN-a, -β and -γ effects and production, anti-apoptotic, transforming
	K11.1	vIRF-2	Blocks IFN-α and -β effects, anti-apoptotic
	K11	?	?
	K10.5/10.6	vIRF-3/LNA-2	Inhibits IFN-α and -β production
	K2	vIL-6	Blocks IFN-α activation of p21CIP1/WAF1, allowing cell cycle progression
	ORF45	KIE-2	Reduces IFN-a production through inhibition of IRF-7 function
	ORF10	RIF	Blocks STAT1, STAT2 and Tyk2 phosphorylation, reducing ISG production
EBV	BCRF1	vIL-10	Blocks IFN-γ and IL-2 production, inhibits dendritic cell maturation, Th2 polarization
	BZLF1	ZTA	Blocks STAT1 activation, MHC class II up regulation and IFN-γ production, anti-apoptotic, disperses PML bodies
	BARF1	vCSF-1R	Blocks IFN-α production, inhibits macrophage proliferation, transforming
	LMP-1/EBI-3	cIL-12 subunit	Alters IL-12 signaling, Th2 polarization, might alter IL-4 and IFN-γ production, might effect NKT cells

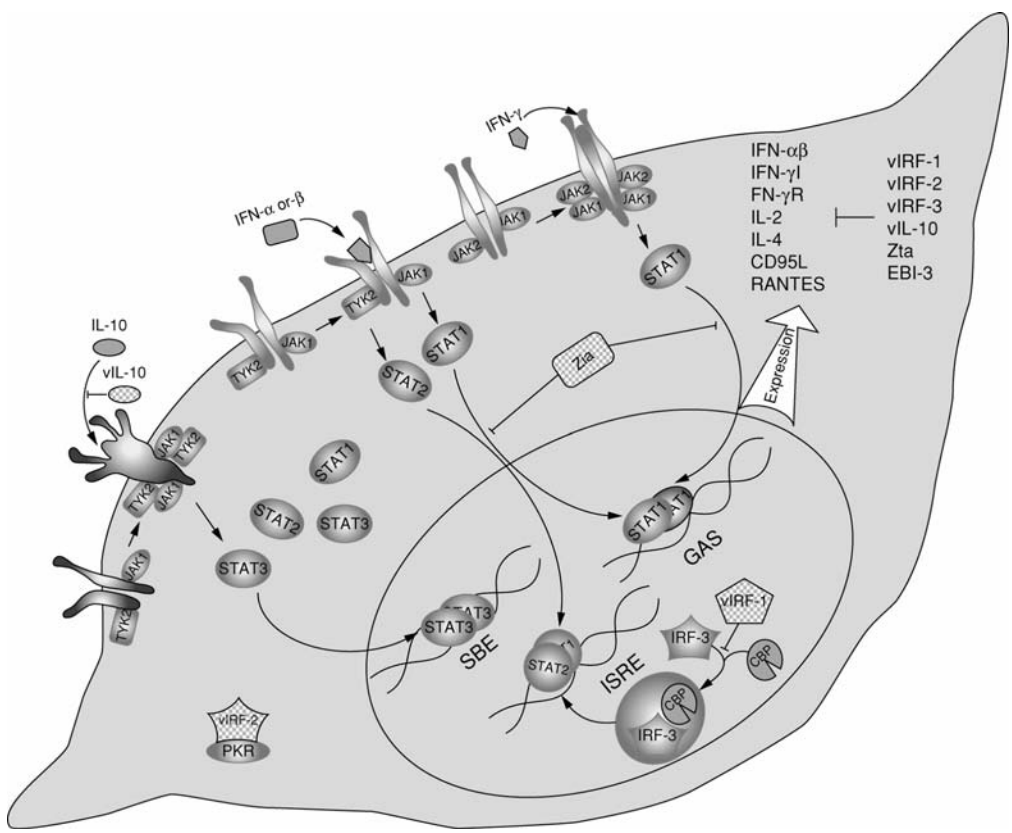


Fig. 31.4. Viral proteins involved in control of the anti-viral IFN responses. A number of γ-HV proteins, detailed in the text and shown in a checkered pattern, are capable of interfering with the IFN responses. Some prevent STAT assembly on GAS, ISRE or STAT binding element (SBE) DNA sequences in the nucleus. Others interfere with the down stream products of the IFN response, such as the binding of PKR by vIRF-2. Additional mechanisms of interference are discussed in the text.

vIRF-1 with the cellular IRF-1 is likely responsible for its ability to suppress CD95L up-regulation and FAS mediated cell death after TCR/CD3 stimulation (Kirchhoff *et al.*, 2002). However, this effect might also be a result of interactions with p300/CBP. More recently, the group of Seo *et al.* (2002) identified an additional binding partner of vIRF-1, retinoid-IFN-induced-mortality-19 (GRIM19). This gene enhances caspase-9 activity and induces apoptosis in response to signals from IFN and retinoic acid treatment of cells. vIRF-1 binding suppresses the ability of GRIM19 to induce apoptosis in HELA and MCF-7 cells. This inhibition of apoptosis through interactions with multiple transcription factors along with an ability to increase c-myc expression all contribute to the transformation ability of vIRF-1.

KSHV encodes three additional vIRFs, vIRF-2, a 489 bp ORF encoded by the ORFK11.1 gene and its close homologue encoded by the ORFK11 gene and vIRF–3, also termed LANA-2, encoded as a 1072 bp spliced product of the ORF K10.5 and 10.6 genes (Burysek *et al.*, 1999; Lubyova and Pitha, 2000). Over expression of these genes results in suppression of both IFN-α and -β signaling (Table 31.3). Unlike vIRF-1, the vIRF-2 protein does not seem to possess transforming activity (Burysek *et al.*, 1999). It is capable of binding to several cellular factors including IRF-1, IRF-2, interferon consensus sequence binding protein, RelA/p65 and CBP (Burysek *et al.*, 1999). vIRF-2 is capable of homodimerizing and binding to DNA encoding the NF-kB sequence (Burysek *et al.*, 1999). More recently it was shown that vIRF-2 is able to interact with the double-stranded RNA-activated protein kinase (PKR) and prevent its autophosphorylation (Burysek and Pitha, 2001). As detailed above, PKR targets the elongation initiation factor 2α (eIF-2α), critical to the initiation of protein synthesis, phosphorylating the GDP-bound inactive form. This phosphorylated form acts as a dominant negative, binding to the eIF-2β guanine nucleotide release factor, preventing the reloading of GTP onto other eIF-2α molecules, thus preventing the initiation of new rounds of protein synthesis (Thomis and Samuel, 1992). All of these abilities of vIRF-2 contribute to its ability to block the effects of the Type I interferons. Intriguingly, vIRF-2 transcripts are present at high levels within 2 hours of viral infection and then subside to undetectable levels by 8 hours post-infection (Krishnan *et al.*, 2004). Further, like vIRF-1, vIRF-2 is able to block FAS-mediated apoptosis through an ability to block up regulation of CD95L on the surface of expressing cells (Kirchhoff *et al.*, 2002).

The transcription pattern of vIRF-3 is more complicated than the other v-IRF genes. Its mRNA is a spliced product of two genomic regions previously thought to code for two separate proteins, ORF K10.6 and ORF K10.5 (Lubyova and Pitha, 2000). It has homology with the vIRF-2 and ORFK11 proteins as well as the cellular IRF-4 (Rivas *et al.*, 2001). It can block the activities of both IRF-3 and IRF-7 on the IFNA promoter, inhibiting the production of both α- and β-interferon (Lubyova and Pitha, 2000). More recent work from Rivas and colleagues has shown that vIRF-3 is able to block PKR-mediated apoptosis, but not oligoadenylate pathway-mediated apoptosis (Esteban *et al.*, 2003). In these studies the expression of vIRF-3 was able to prevent PKR mediated inhibition of protein synthesis, at least partially through a block to the phosphorylation of eIF-2α. Further, vIRF-3 was able to block the activation of caspase 3, a member of the FADD/caspase 8 pathway of apoptosis that is activated by PKR. However, no effects of vIRF-3 were observed on caspase 9, another PKR activated caspase. Since vIRF-2 also targets the PKR pathway, an exploration of the co-expression of these genes and their potentially additive effects on antiviral interferon responses would be interesting.

More recently, in a comprehensive screen of the KSHV lytic genes, Ganem's group identified a viral protein capable of blocking interferon signaling at a membrane proximal position, unlike the majority of KSHV gene products that block responses in the nucleus. ORF 10, now named RIF (regulator of Interferon Function), is able to block phosphorylation of STAT1, STAT2 and Tyk2 following IFN-α stimulation (A-L Page, SA Bisson and D Ganem, personal communication). Interestingly, this viral protein directly interacts with the STAT proteins, potentially blocking their multimerization. This mechanism seems distinct from that of the EBV BZLF1 gene product ZTA, which will be discussed in detail later in this section, as ZTA only blocks STAT1 phosphorylation following IFN-γ stimulation, not IFN-α (Morrison *et al.*, 2001).

The interferon responses typically take place very quickly after viral infection. Therefore, in order to persist within the host the virus must take immediate protective action. Zhu *et al.* have identified a KSHV protein encoded by ORF45 that is incorporated into the virus particle as a tegument protein (Zhu and Yuan, 2003). This protein, KIE-2, is able to bind to IRF-7, blocking its phosphorylation and accumulation in the nucleus (Zhu *et al.*, 2002). This results in a blockage of IFN-α and IFN-β transcription in response to viral infection. As its name suggests, KIE-2 is an immediate early protein of ~78kDa and is found within the cytoplasm of expressing cells. Based on the presence of the protein in preparations of purified virus and its resistance to detergent treatment combined with sensitivity to detergent plus trypsin suggests that this protein is found in the tegument of the virus. This protein, therefore, would be available to block the interferon responses immediately following viral infection.

KSHV possess yet an additional way of mitigating the effects of interferon on viral replication and persistence. The viral IL6 homologue is able to block the induction of $p21^{CIP1/WAF1}$, a cyclin-dependant kinase inhibitor, by IFN-α (Table 31.3). Normally treatment of cells results in an up regulation of this protein, arresting the cells in G_1/S (Chatterjee *et al.*, 2002). Further, treatment of cells with vIL-6 was shown to block the IFN-α stimulated binding of ISGF3 to ISRE probes. Interestingly, IFN-α downregulates the gp80 sub-unit of the IL-6 receptor blocking the ability of cIL-6 to transmit a signal. On the other hand, vIL-6 only requires the gp130 sub-unit to signal and thus, is not blocked by the down regulation of gp80. Additionally, vIL-6 expression is induced by IFN-α, providing a negative feedback loop to control this antiviral response in virally-infected cells.

One remaining question is why seven different gene products potentially involved in regulating the cellular IFN responses are encoded by the virus. Several non-exclusive answers exist. First, although each of the studied products possess similar functions, they are not identical. Given the importance of the IFN response in the control of other viruses, as well as a proven ability of interferon treatment to block KS progression in a large percentage of patients, this simplistic answer is probably also true (Von Roenn and Cianfrocca, 2001). Recent work by a number of groups using DNA array technology has pointed to another potential answer. When examining expression of each vIRF gene, it was found that the kinetics and tissue-expression of each differed (Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001; Fakhari and Dittmer, 2002; Dittmer, 2003). Unlike vIRF-1, the vIRF-3/LANA-2 protein is detectable in primary effusion lymphoma (PEL) cell lines without TPA stimulation. The work of Fakhari and Dittmer demonstrated that the kinetics of vIRF-3/LANA-2 mRNA production mirrored that of LANA-1, v-FLIP and v-cyclin, all non-TPA induced, latency-associated genes in the BCBL-1 PEL cell line (Fakhari and Dittmer, 2002). In KS lesions, however, expression of vIRF-1 and not vIRF-3 clusters with LANA-1. So, a degree of tissue- or disease-specific expression might also contribute to the need for multiple genes to combat this facet of innate immunity. An understanding of the role of the vIRFs during infection is further complicated by the work of Pozharskaya *et al.* (2004). In experiments looking at vIRF-1 it was found that during latency in BCBL-1 PEL cells, only low levels of vIRF-1 are expressed and are not able to block the effects of IFN-α and while higher levels were initially expressed after TPA induction, these levels quickly fell off. Further, the vIL-6 gene is transcribed to high levels shortly after infection (Krishnan *et al.*, 2004). In summary, KSHV expresses multiple genes capable of blunting the production and effects of the interferon genes. These genes are expressed during both the lytic and latent programs, underlining the importance of the interferon proteins in the control of viral infection.

Like KSHV, EBV encodes multiple genes that help it avoid the antiviral effects of interferon. Among these are BCRF1, BZLF1 and BARF1 (Table 31.3). Additionally, viral infection induces the expression of a cellular protein named EBI-3, also involved in regulating the effects of interferon. The mature BCRF1 gene product (vIL-10) possesses 84% identity with cellular interleukin-10 (Hsu *et al.*, 1990). A 170 residue protein, it is expressed late in the lytic program, although one group reports expression of BCRF1 in a small number of patients with nasal type, extranodal natural killer (T(NK/T)-cell) lymphoma, which is usually associated with latent EBV infection (Swaminathan *et al.*, 1993; Xu *et al.*, 2001). This gene product indirectly effects the production of the type II IFN-γ by binding to the cellular IL-10 receptor. This results in a blockage of IL-2 and IFN-γ production (Liu *et al.*, 1997; Takayama *et al.*, 2001). Like cellular IL-10, vIL-10 can block the maturation of dendritic cells causing them to down regulate CCR7 and up regulate CCR5. This blunts their ability to stimulate T cell release of IFN-γ (Takayama *et al.*, 2001). In addition to aiding the virus in escape from IFN responses this has profound effects on CTL induction, which will be discussed in a later section of this chapter. Viruses containing a truncated BCRF1 protein or completely deleted for the gene were functionally similar to wild-type virus (Swaminathan *et al.*, 1993). Like the parental virus, BCRF1 deleted virus was able to transform B-lymphocytes into long-term lymphoblastoid cell lines (LCLs), and these LCLs were capable of inducing tumors in SCID mice to the same degree as wild-type derived LCLs (Swaminathan *et al.*, 1993). This suggests that BCRF1 is playing a larger role in immune regulation than in viral pathogenesis.

The BZLF1 gene expresses an immediate early viral protein with a wide number of functions. Not only is it important in directing the lytic replication program through its binding to the lytic origin of replication and within several of the early lytic gene promoters, but it also blocks tyrosine phosphorylation and nuclear translocation of STAT1, an important molecule in IFN response signaling (Kenney *et al.*, 1989; Rooney *et al.*, 1989; Packham *et al.*, 1990; Morrison *et al.*, 2001). Additionally, this protein blocks IRF-1 activation and decreases the amount of IFN-γ α-chain receptor expression (Morrison *et al.*, 2001). BZLF1 is able to decrease the ability of IFN-γ to activate a variety of important downstream target genes, such as IRF-1, p48, and CIITA, and prevents IFN-γ-induced class II MHC surface expression (Morrison *et al.*, 2001). Like BCRF1, not only does BZLF1 affect interferon responses, but it also

interferes with activation of helper T-cells and it possesses anti-apoptosis activity, both of which will be discussed in greater detail in later sections of this chapter. Interestingly, through competition for limiting amounts of the SUMO-1 protein, BZLF1 can also disperse nuclear PML bodies, which are induced by interferon and posited to have antiviral effects (Adamson and Kenney, 2001).

The BARF1 gene encodes a 31–33 kDa soluble receptor for colony stimulating factor 1 (CSF-1) that is expressed as an early lytic gene (Wei and Ooka, 1989; Strockbine *et al.*, 1998). Its expression inhibits macrophage proliferation and blocks IFN-α production by monocytes (Cohen and Lekstrom, 1999). Additionally, BARF1 can act as an oncogene when expressed in fibroblasts, B-lymphoma cells and monkey kidney cells (Wei and Ooka, 1989; Wei *et al.*, 1994, 1997). It increases c-myc, CD21 and CD23 expression and introduction into EBV^- Akata cells resulted in increased Bcl-2 expression and tumor induction in SCID mice (Wei *et al.*, 1994; Sheng *et al.*, 2001, 2003). However, Cohen and Lekstrom (1992) demonstrated that a $BARF1^-$ virus was competent for B cell transformation. Groups have reported that in both gastric adenocarcinomas and NPC, BARF1 is strongly expressed along with a number of the other latent proteins (Hayes *et al.*, 1999; Decaussin *et al.*, 2000; zur Hausen *et al.*, 2000). In the case of EBV positive gastric adenocarcinomas, this is in the absence of the LMP1 oncogene, giving greater weight to the ability of BARF1 to act as an oncogene, at least in certain tissues or cell-types. Fewer reports have been made concerning the potential immune evasion role of BARF1.

One final gene utilized by EBV to control the anti-viral interferon responses is encoded by the host and induced by the LMP1 protein (Devergne *et al.*, 1996, 1998). EBV-induced gene-3 (EBI-3) is a 34 kDa glycoprotein which localizes primarily to the ER of expressing cells (Devergne *et al.*, 1996). It is homologous to the IL-12 p40 subunit and can bind to IL-12 p35 (Devergne *et al.*, 1996). IL-12 normally triggers Th1 polarization of naïve $CD4^+$ T-cells, which then secrete IFN-γ. Recently, Pflanz and coworkers (Pflanz *et al.*, 2002) demonstrated that IL-27 is made up of a complex of EBI-3 and IL-12 p35. This interleukin is produced by activated antigen presenting cells and can drive expansion of naïve $CD4^+$ T cells, although the effects on EBV are not yet clear since IL-12 can synergize with IL-27 for IFN-γ production and effect both CTL and NK cell development (Pflanz *et al.*, 2002). An EBI-3-knockout mouse has normal numbers of most immune effectors except invariant natural killer T cells. This results in decreased IL-4 production and some decreases in IFN-γ production. The cellular EBI-3 protein, therefore, is playing a critical role in the generation of Th2 immune responses and its induction by EBV infection probably drives polarization of the anti-viral immune response (Nieuwenhuis *et al.*, 2002).

The LMP1 protein makes at least one potential additional contribution to viral avoidance of the interferon defenses. Quizzically, LMP1 induces the expression, activation and nuclear translocation of IRF-7, the same IRF that the KSHV ORF45 gene product inactivates (Zhang and Pagano, 2001; Zhang *et al.*, 2001). It has been shown that expression of LMP-1 in cells induces a number of ISGs and can block the replication of vesicular stomatitis virus (Zhang *et al.*, 2004). The paradoxical stimulation of what would seem to be an antiviral state within the cell most likely plays a role in controlling EBV latency and superinfection of EBV^+ cells by other viruses.

Thus, like KSHV, EBV encodes numerous proteins capable of altering the antiviral interferon responses. These proteins are expressed at multiple time points during the viral life cycle, highlighting how important the interferon responses are for the control of viral infections. Further examination of these groups of genes in an in vivo context should yield important information about how the interferon responses are modulated within the host.

Apoptosis responses

Apoptosis or programmed cell death plays a role both in innate immunity and normal cellular regulation. It is a mechanism by which intrinsic or extrinsic signals are capable of inducing cell death for the purposes of removing a diseased or unwanted cell from the body. Central to the intrinsic apoptotic responses are the members of the BCL-2 protein family. These proteins are capable of either inducing or suppressing apoptosis and possibly function through homo- or hetero-dimerizing with other family members, although this is controversial. The extrinsic responses, such as those triggered by $CD8^+$ CTL, largely depend on members of the tumor necrosis factor (TNF) receptor family. These receptors contain death response domains in their cytoplasmic tails and upon multimerization transmit a signal to the intracellular caspases that initiate the apoptosis response. It is critical that the γ-HV control apoptosis in order to insure that the infected cell is not eliminated prior to virion production.

Both EBV and KSHV encode homologues of the cellular Bcl-2 gene (Table 31.4). The KSHV Bcl-2 homologue (vBcl-2) is expressed from ORF16, but only possesses low homology with cellular Bcl-2 (Russo *et al.*, 1996; Neipel *et al.*, 1997a,b). Little is known of the mechanism of action of this protein. While it can inhibit Bax toxicity in yeast and fibroblasts, there is conflicting data concerning its ability to dimerize with the cellular Bcl-2 family members (Cheng

Table 31.4. Viral apoptosis regulators. Cellular suicide, whether self-induced or induced by other effectors, is an important immune response to control the replication and spread of viruses. Both human γ-herpesviruses encode multiple gene products with the potential to alter the cell suicide, apoptosis response. The open reading frame, product and potential function are listed for each, with additional details in the text

	Gene	Product	Function
HHV-8	Orf16	vBcl-2	blocks both Bax and vCyclin induced apoptosis
	Orf13	vFLIP	up regulates Bcl-x(L) through the NF-kB pathway blocking starvation-mediated apoptosis, up regulates IL-6 in a TRAF2/JNK/AP1 dependent fashion, blocks Fas-mediated apoptosis, interferes with caspase-3, -8 and -9, tumorigenic
	K9	vIRF-1	binds IRFs, GRIM19, p53 and p300/CBP, blocks p53 and IFN + retinoic acid induced apoptosis
	K7	K7/vIAP	binds CAML, cBcl-2 and caspase-3, preserves mitochondrial membrane potential, blocks TRAIL, staurosporin and thapsigargin-induced apoptosis
EBV	BHRF1	vBcl-2	preserves mitochondrial membrane potential, blocks TRAIL and Fas-mediated apoptosis
	BCRF1	vBcl-2	binds HAX-1 and Bcl-2 through EBNA-LP effecting apoptosis
	BALF1	vBcl-2	binds Bax and Bak, can modulate BHRF1 activity
	BNLF1	LMP-1	increases Bcl-2, A20 and Traf1 expression blocking TNF-mediated apoptosis, can induce bfl-1 blocking p53-mediated apoptosis

et al., 1997; Sarid *et al.*, 1997). Additionally, vBcl-2 is capable of blocking the apoptosis induced by viral cyclin, so whether KSHV vBcl-2 is acting to protect virally infected cells against the extrinsic pro-apoptotic immune responses or intrinsic virally-mediated apoptotic responses is unclear (Ojala *et al.*, 1999, 2000).

In addition to a Bcl-2 homologue, KSHV also encodes a homologue of the cellular FLICE inhibitory protein, termed vFLIP (Chang *et al.*, 1994). It is expressed from ORF13 as a multi-cistronic transcript with ORF72 (vCyclin) and ORF73 (LANA) through either differential splicing or expression from an IRES element (Sarid *et al.*, 1999; Grundhoff and Ganem, 2001; Low *et al.*, 2001). FLICE, or caspase-8, is a member of the ICE family of cellular caspases, and is important in the apoptosis response (Muzio *et al.*, 1996). Interestingly, all three of these genes are transcribed rapidly after infection, underscoring a potential need to combat the apoptotic response soon after viral entry into cells (Krishnan *et al.*, 2004). The vFLIP protein has been shown to block pro-apoptotic signaling mediated by the Fas-receptor, resulting in decreases in caspase-8, -9 and –3 activity (Thome *et al.*, 1997; Djerbi *et al.*, 1999; Belanger *et al.*, 2001). A recent study has shown that vFLIP can target the NF-kB pathway, up regulating Bcl-x(L) resulting in protection of cells from serum withdrawal (Sun *et al.*, 2003). Additionally, vFLIP physically interacts with tumor necrosis factor receptor associated factor 2 (TRAF2) activating the JNK/AP1 pathway in a TRAF-dependent fashion (An *et al.*, 2003). This modulation of the JNK/AP1 pathway results in the induction of IL-6, important in directing a Th2 polarization of the immune response and having proliferative effects as detailed earlier (An *et al.*, 2003). Further, vFLIP expression promoted tumor formation after injection of syngeneic and semiallogeneic mouse strains with A20 cells expressing this protein (Djerbi *et al.*, 1999).

The K9/vIRF-1 protein, able to block the anti-viral interferon responses as described above, also has a role in blocking programmed cell death. In addition to binding cellular IRF and GRIM19, vIRF-1 has been shown to bind both p53 and p300/CBP through tryptophan- and proline-rich sequences (Li *et al.*, 2000; Seo *et al.*, 2000; Nakamura *et al.*, 2001; Seo *et al.*, 2001). The irreversible cell cycle arrest and cell death induced by p53 are considered part of host surveillance mechanisms for detecting and preventing viral infection and tumor induction. The activity of p53 is regulated by a series of kinases, phosphatases and acetylases. Acetylation of the carboxyl-terminal region of p53 is mediated by p300 and p300/CBP-associated factor (PCAF) (Sakaguchi *et al.*, 1998). This modification leads to increased DNA binding activity. Interactions of vIRF-1 with p53 and p300/CBP lead to decreased acetylation of p53 as well as decreased phosphorylation, resulting in a dramatic decrease in p53 activity (Nakamura *et al.*, 2001). Blocking p53-dependent transcription suppresses Bax and p21 transcription, mediators of p53-mediated apoptosis, thus rescuing vIRF-1 expressing cells from programmed cell death (Nakamura *et al.*, 2001; Seo *et al.*, 2001). Additionally, interactions of vIRF-1 with CBP results in hypoacetylation of histones H3 and H4, reducing transcription from the early inflammatory gene promoter (Li *et al.*, 1998).

KSHV encodes at least one additional protein that has anti-apoptotic activity. The K7 protein, also termed vIAP, localizes to the mitochondria of expressing cells where it can interact with the cellular calcium-modulating

cyclophilin ligand (CAML) (Feng *et al.*, 2002; Wang *et al.*, 2002). This interaction helps to maintain the mitochondrial membrane potential after treatment with a variety of pro-apoptotic compounds including TRAIL, staurosporin and thapsigargin (Feng *et al.*, 2002). Additionally, K7 has been shown to interact with the Protein-linking integrin-associated protein and cytoskeleton 1 (PLIC1) (Feng *et al.*, 2004). PLIC1 is capable of dimerizing and binding to poly-ubiquitin containing proteins, as well as associating with the 19s unit of the proteasome (Feng *et al.*, 2004). KSHV K7 is able to reduce the ability of PLIC1 to homodimerize and bind to ubiquitinylated proteins, with the end result that two of the targets of PLIC1 activity, Iκb and p53, are rapidly degraded in the presence of K7 (Feng *et al.*, 2004). This reduction of p53 levels within the cell contributes to the anti-apoptotic action of K7. Finally, the K7 protein has also been shown to interact with cellular Bcl-2 and caspase-3, but not with Bax and these interactions are critical to its anti-apoptotic activity (Wang *et al.*, 2002). Like the vFlip gene, K7 is transcribed rapidly after viral infection (Krishnan *et al.*, 2004).

The EBV BHRF1 protein is an early lytic protein capable of blocking the pro-apoptotic actions of TNF-related apoptosis inducing ligand (TRAIL) and FAS (Cheng *et al.*, 1997; Foghsgaard and Jaattela, 1997; Kawanishi, 1997; Kawanishi *et al.*, 2002). TRAIL normally causes cleavage of Bid, a BCL-2 family member, via activation of caspase 8. BHRF1 doesn't block Bid cleavage, but it does block loss of mitochondrial membrane potential, an important downstream apoptotic effect (Kawanishi *et al.*, 2002). Recently, the NMR structure of BHRF1 was solved, demonstrating some clear differences with the structure of its cellular counter-part (Huang *et al.*, 2003). Unlike Bcl-2, it does not contain a hydrophobic groove important in homo- or hetero-dimerization with other apoptotic factors. Additionally, BHRF1 doesn't bind to peptides from Bak, Bax, Bik, and Bad, indicating it functions in a fundamentally different way than the cellular Bcl-x(L) or Bcl-2 (Kawanishi *et al.*, 2002; Huang *et al.*, 2003). BCRF1 protein is also able to complex with another EBV protein, EBV nuclear antigen leader protein (EBNA-LP) (Matsuda *et al.*, 2003). Previously it was shown that EBNA-LP can bind to the HS1-associated protein X-1 (HAX-1), while more recently it was shown that this protein can bind to cellular Bcl-2 (Kawaguchi *et al.*, 2000; Matsuda *et al.*, 2003). The implications of this complex web of interactions to apoptosis is made more complicated by a third EBV protein, BALF1. BALF1 is also an early lytic, Bcl-2 homologue (Hatfull *et al.*, 1988). It can interact with both the Bak and Bax BCL-2 family members and was originally demonstrated to have apoptotic effects (Marshall *et al.*, 1999). More recent experiments have shown that BALF1 can block the action of BHRF1 through an unknown mechanism, but itself has no direct pro- or anti-apoptotic activity (Bellows *et al.*, 2002).

The LMP-1 gene of EBV is expressed during latency and has been shown, in addition to its latency regulatory activity, to prevent apoptosis. It can increase the expression of cellular Bcl-2 as well as a number of other cellular proteins including A20 and TRAF1, important in the anti-apoptotic TNF receptor pathway, through its activation of NF-κB (Henderson *et al.*, 1991; Devergne *et al.*, 1998). LMP-1 is additionally able to induce the expression of bfl-1, a Bcl-2 homologue able to suppress p53 mediated apoptosis (D'Souza *et al.*, 2000). Ectopic expression of bfl-1 in an EBV-positive cell line exhibiting a latency type I infection protects against apoptosis induced by growth factor deprivation, thereby providing a functional role for bfl-1 in this cellular context and adding bfl-1 to the list of anti-apoptotic proteins whose expression is modulated by EBV.

As outlined in this section, the γ-HVs encode a large number of proteins aimed at controlling the cellular apoptosis response. These proteins likely aid the virus in escape from immune effectors such as NK cells and CTL, as well as preventing viral replication from inducing cell death. Given the central importance of programmed cell death in immune function, additional viral mechanisms to avoid apoptosis are likely to be discovered.

Natural killer (NK) cell responses

The NK cells play a critical role in clearing virally-infected cells through direct lysis and the release of various cytokines, which coordinate other immune responses. Although the task that they perform is simple, their regulation is not. A complex set of cell:cell interactions determine whether the NK cell will release its deadly cargo of perforin and granzyme to induce programmed cell death in the target cell, secrete large amounts of IFN-γ to stimulate Th1 T cell production or release the target cell unharmed. The cell surface receptors that govern NK activity can be split into four classes (for review see Anderson *et al.*, 2001; Boyington *et al.*, 2001; LaBonte *et al.*, 2001; Long *et al.*, 2001; McVicar and Burshtyn, 2001; Volz *et al.*, 2001). The killer cell immunoglobulin receptors (KIR) make up the first class. They are capable of binding to a variety of MHC class I haplotypes and generally transmit a negative signal. The second class is the C-type lectin receptor family composed of heterodimers of CD94 and one of several NKG2 proteins. Like the KIR, these receptors also bind MHC class I molecules, but only HLA-G and –E. The natural cytotoxicity receptors (NCR) compose the third class of receptors. The NCR don't interact with class I, but as of yet no cognate ligands have been identified. All identified NCR transmit

Table 31.5. Viral NK regulators. The immune system employs a number of specialized cellular effectors that perform general "house-keeping" function, including the elimination of diseased cells. The natural killer cells are a sub-set of these effectors that are capable of sensing the health of a cell through a number of cell surface receptors, including several which recognize MHC class I. Both human γ-herpesviruses encode gene products with the potential to alter the response of NK cells to infection. The open reading frame, product and potential function are listed for each, with additional details in the text

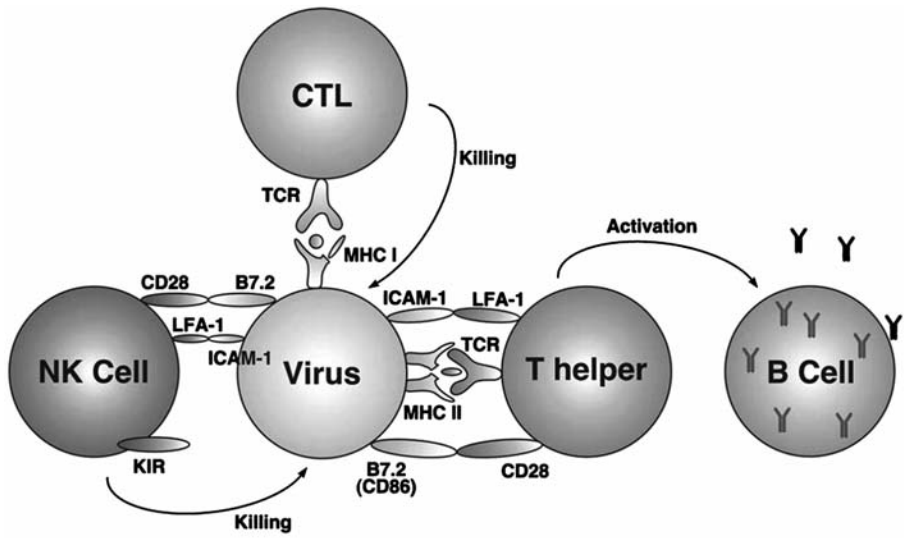
	Gene	Product	Function
HHV-8	K5	K5 (MIR-2)	Down regulates HLA-A and –B, B7.2 and ICAM-1, but leaves HLA-C on the surface of cells blocking NK cell lysis
EBV	LMP-1/EBI-3	cIL-12 subunit	Stabilizes HLA-G1 surface expression sending a negative signal to the NK cells

positive signals to their expressing NK cells, inducing killing and cytokine release in the absence of stronger negative signals. The fourth class, the leukocyte immunoglobulin-like receptor (LIR) family, similar to the class two receptors, can bind HLA-G to transmit a negative signal. The net overall strength of each positive and negative signal determine whether the NK cell will be turned on to make a response or induced to release the target cell.

Important to NK surveillance are multiple adhesion molecules on both the NK and target cell. These molecules include the integrins, intracellular adhesion molecule 1 (ICAM-1), CD2 and LFA3. Interactions between these molecules help to bring the NK cell into close conjugation with its target. This allows both for the NK cell to survey the target cell for the many positive and negative regulatory factors and to potentially deliver the perforin/granzyme payload specifically to the closely juxtaposed target. Antibodies that block the binding of the NK adhesion molecules to the target cell have been shown to block NK cell lysis (Papa *et al.*, 1994; Komatsu and Kajiwara, 1998). Experiments from Burshtyn *et al.* demonstrated that when the KIR interacts with MHC class I on the target cell and transmits a negative signal, there is a decrease in the ability of the NK cell to stay in conjugation with the target (Burshtyn *et al.*, 2000).

The KSHV K5 protein is capable of inducing the down regulation of several cell surface proteins through increasing their rate of endocytosis. This protein will be further discussed later, but as a brief introduction, K5 seems to act as an E3 ubiquitin ligase, targeting ICAM-1, B7.2 and some MHC class I haplotypes for destruction by the ubiquitin: proteasome system (Coscoy and Ganem, 2000; Ishido *et al.*, 2000a,b; Coscoy and Ganem, 2001; Coscoy *et al.*, 2001; Means *et al.*, 2002; Sanchez *et al.*, 2002). This destruction happens in a sequential manner with the targeted proteins first being endocytosed from the cell surface into the trans-Golgi network (Means *et al.*, 2002). Target proteins are then redirected into the lysosome, where they undergo destruction (Means *et al.*, 2002). While MHC I normally acts to transmit a negative signal to NK cells, the ICAM-1 and B7.2 molecules act as anchors to bring the NK cell into close conjugation with target cells. By removing these last two molecules from the surface of infected cells, K5 reduces the average time that the NK cell stays in contact with the K5-expressing target cell (Table 31.5). Thus, even without MHC class I present to transmit a negative signal through the KIR, the NK cell releases the K5-expressing cell unharmed, simply because it can't stay in contact long enough to get a strong positive signal and turn on granzyme/perforin or cytokine release (Ishido *et al.*, 2000a,b) (Fig 31.5).

Less information is available concerning the avoidance of NK cell responses by EBV. The work of Devergne *et al.* (2001) demonstrated that the EBV-induced EBI-3 protein is capable of stabilizing HLA-G presentation on the surface of cells (Table 31.5). HLA-G1 is capable of transmitting a negative signal to NK cells, preventing the activation of killing or cytokine elicitation (Adrian Cabestre *et al.*, 1999; Navarro *et al.*, 1999; Rajagopalan and Long, 1999). Further, HLA-G1 expression was shown to block the lysis of cells presenting HLA-A2-restricted influenza epitopes to specific CTL clones (Le Gal *et al.*, 1999). However, no experimental data has been presented to demonstrate that the LMP1-mediated up regulation of EBI-3 is able to convey these immune avoidance phenotypes in the context of EBV infection (Fig 31.5). One other mechanism that EBV may be using to avoid NK surveillance is through direct infection. While NK cells lack the CD21/CR2 EBV receptor, studies have shown that they become briefly $CD21^+$ after conjugation with $CD21^+$ B-cell targets (Tabiasco *et al.*, 2003). Acquisition of this molecule allows for EBV infection and likely underlies the genesis of EBV^+ NK cell lymphomas, while at the same time allowing for viral escape from NK surveillance.



NK Cell

Gene	Modulation target
K5	ICAM-1
LMP-1	HLA-G1

CTL

Gene	Modulation target
K3	MHC I
K5	MHC I
BCRF1	TAPI → MHC I

T-helper and B-cell

Gene	Modulation target
K5	B7.2
	ICAM-1
BZLF1	MHC II

Fig. 31.5. Viral alteration of immunomodulatory proteins. Through the down regulation of multiple immunomodulatory proteins both KSHV and EBV are able to alter the ability of innate and adaptive immune effectors to recognize and mount responses against virally infected cells. Diagramed are some of the cellular proteins altered by viral gene products and their binding partners. The viral proteins and their targets are listed in the tables below the diagram. Details of the interactions are given in the text.

While it is clear that both of the human γ-HV interfere with NK cell surveillance the possibility of additional viral mechanisms for avoiding these effectors remains. For example, the human cytomegalovirus (HCMV), a β-herpesvirus, encodes the UL16 protein. This protein is able to bind and down regulate the cellular UL16-binding proteins (ULBP) 1 and 2, which are ligands for the c-type lectin NKG2D activating receptor on NK cells (Rolle *et al.*, 2003). This effectively protects HCMV-infected cells from NK cell lysis. To date, no human γ-HV protein has been shown to target an NK cell activating receptor.

Evasion of other innate cellular responses

The immune system employs a number of additional innate effectors which surveil the body, including the professional phagocytes, neutrophils and macrophage that internalize and destroy extracellular pathogens such as bacteria or virus, as well as eosinophils and basophils, both able to release a number of immunomodulatory proteins. Like NK cells, these cells represent the first line of defense against infecting pathogens and are able to modulate the later adaptive responses. It is therefore critical that the γ-HV deregulate or avoid recognition by these cells in order to establish a persistent infection.

EBV is able to infect both neutrophils and monocytes, macrophage-precursors. While EBV infection of neutrophils is abortive, multiple changes in cellular physiology important to the potential escape of the virus from immune avoidance occur (Larochelle *et al.*, 1998). First, viral infection causes the up regulation of Fas ligand. Neutrophils express CD95/Fas, which plays a role in immune privilege, and the increased expression of Fas ligand results in apoptosis (Griffith *et al.*, 1995; Larochelle *et al.*, 1998). This effectively eliminates responding neutrophils. However, EBV infection also triggers these cells to release a number of cytokines, including IL-8, MIP-1α, IL-1α, IL-1β and the IL-1R antagonist (Beaulieu *et al.*, 1995; McColl *et al.*, 1997; Roberge *et al.*, 1997). These chemokines, along with the highly-induced leukotrien B4, act to recruit additional leukocytes, potentially aiding in viral dissemination (Gosselin *et al.*, 2001).

Table 31.6. Viral CTL Regulators. $CD8^+$ T-cell responses play a critical role in the elimination of virally-infected cells. Both human γ-herpesviruses encode multiple gene products with the potential to alter the ability of host CTLs to recognize and eliminate virally-infected cells. The open reading frame, product and potential function are listed for each, with additional details in the text

	Gene	Product	Function
HHV-8	K3	K3 (MIR-1)	Down-regulates MHC class I, blocking induction of CTL responses
	K5	K5 (MIR-2)	Down-regulates HLA-A and -B, B7.2 and ICAM-1 blocking CTL and T helper activation
	LANA	LANA	Block *in cis* CTL epitope presentation
EBV	BKRF1	EBNA-1	GAR region interferes with the proteasome and MHC class I presentation
	BCRF1	vIL-10	Down regulates TAP1 and cLMP2 interfering with MHC class I loading and presentation

In contrast, infection of monocytes, while highly inefficient, seems to be productive, resulting in transformed monocyte cell lines displaying type II latency (Masy *et al.*, 2002). The route of infection is unclear since monocytes do not express detectable levels of CD21, however, transient CD21 expression might result from the engulfment of $CD21^+$ cells (Inghirami *et al.*, 1988). Addition of the gp350/220 glycoprotein of EBV to monocytes results in the elicitation of IL-1, IL-6 and TNF-α (D'Addario *et al.*, 1999; 2000). However, addition of the whole virus does not up regulate IL-1 expression and blocks TNF-α secretion (D'Addario, 1999; Gosselin *et al.*, 2001). Finally, EBV infection has been shown to decrease the production of prostaglandin E2 (Savard *et al.*, 2000). This would be expected to induce or favor a Th1 response, but in the face of other virally-elicited cytokines, it probably acts to elicit an inflammatory response, recruiting additional targets for viral infection.

These alterations in innate cellular responses along with the alterations in eosinophils function described earlier likely all contribute to long-term control of the anti-viral immune responses. By controlling the cells which initially determine the direction the immune response is to take, the γ-HV are able to insure that they are able to establish a persistent infection. Using these cells, the virus is able to skew responses made by the immune effectors such that the additional immunomodulatory genes are most effective.

Evasion of adaptive host immunity

The adaptive immune responses are mediated by the $CD4^+$ and $CD8^+$ T-cells and the antibody producing B-cells. Like the innate responses, there are multiple levels of regulation and therefore, multiple opportunities for viral intervention. The adaptive immune responses provide two critical "improvements" over the innate responses: the memory response, allowing the immune system to react more quickly and effectively to a previously seen pathogen, and response maturation, allowing for a more targeted, higher affinity response to a pathogen. These differences from the innate responses can also be taken advantage of by invading pathogens in the form of dominant epitopes or antigenic variation, both of which can mislead the immune system into making ineffective responses.

Evasion of CTL responses

The cytotoxic T-lymphocytes are $CD8^+$ T cells that can directly lyse and induce apoptosis in infected cells, as well as releasing cytokines such as IFN-γ, TNF-α and TNF-β. Presentation of non-self antigens in complex with MHC class I on the surface of infected cells along with co-stimulatory molecules can activate this killing and the γ-HVs have devised several ways of preventing this from occurring as outlined in Table 31.6.

KSHV encodes two genes, K3 (MIR-1) and K5 (MIR-2), whose products are able to down regulate MHC class I from the surface. Respectively, they are the eleventh and fourteenth Orfs from the left end of the genome, encoding products with approximately 40% identity (Russo *et al.*, 1996). They are early lytic proteins and showed increased expression in TPA treated PEL cells (Sun *et al.*, 1999). Work from Krishnan *et al.*, (2004) demonstrated that the K5 gene product is also expressed very rapidly after infection with KSHV. The levels of this protein then slowly decline over the next several days. The K3 and K5 proteins are both type III integral membrane proteins containing a zinc-binding Really Interesting New Gene (RING-CH) sequence at the N-termini, two hydrophobic transmembrane regions and a series of protein motifs important in cellular trafficking in the C-terminus (Coscoy and Ganem, 2000; Ishido *et al.*, 2000; Means *et al.*, 2002; Sanchez *et al.*, 2002). Both have been shown to insert into the ER membrane such that the N- and C-termini are projecting into the cytosol (Sanchez *et al.*, 2002). The PHD domains of these proteins resemble

those found in a number of E3 ubiquitin ligases and are capable of mediating self ubiquitinylation when fused to the GFP protein (Coscoy *et al.*, 2001). While the exact mechanism of MHC class I molecule down regulation is still only partially understood, this ability of K3 and K5 to act as an E3 ligase seems critical. The transmembrane regions of these two proteins probably play at least two roles. First, they define the target specificity. K3 is able to down regulate multiple HLA haplotypes, whereas K5 down-regulates a much more restricted set. However, K5 is additionally able to target the cellular B7.2 costimulatory molecule and ICAM-1 adhesion molecule for down-regulation (Coscoy and Ganem, 2000; Ishido *et al.*, 2000; Means *et al.*, 2002; Sanchez *et al.*, 2002). The selection of targets is regulated by the transmembrane domains (Sanchez *et al.*, 2002). Second, these sequences probably allow K3 and K5 multimerization, although it isn't clear what role this plays in their function (Sanchez *et al.*, 2002). Downstream of the transmembrane regions, both contain a conserved series of residues identified as being important in protein:protein interactions and cellular trafficking (Means *et al.*, 2002; Sanchez *et al.*, 2002). Several of these motifs, including a Y–X–X–ϕ endocytosis sequence, direct internalization of the target proteins from the cell surface into the trans-Golgi network (TGN) (Means *et al.*, 2002). From there other motifs, primarily two stretches of acidic amino acids, redirect the target proteins to the endosomal/lysosomal compartment where they undergo destruction by the ubiquitin:proteasome system (Lorenzo *et al.*, 2002; Means *et al.*, 2002). Without MHC class I on the cell surface, no peptides are presented to induce CL activation and K3/K5 expressing cells are able to escape killing (Ishido *et al.*, 2000) (Fig. 31.5). Down regulation of ICAM-1 likely also reduces the non-specific surveillance of cells by $CD8^+$ effectors.

EBV also regulates MHC class I presentation of viral peptides. EBV nuclear antigen (EBNA)-1 is a latent viral protein and contains a glycine, alanine repeat (GAR) region and plays a critical role in maintenance and segregation of the viral episome (Hennessy and Kieff, 1983; Yates *et al.*, 1984). The GAR region inhibits proteasome functioning, greatly decreasing presentation of EBNA-1 peptides derived from full-length protein, as well as limiting EBNA-1 mRNA translation (Levitskaya *et al.*, 1997; Yin *et al.*, 2003; Blake *et al.*, 1997). This has the effect of keeping the levels of EBNA-1 low, but stable, in latently infected cells. In addition to proteasome-independent presentation of EBNA-1 peptides, most likely by professional APCs that take up dead or dying EBV-infected cells, EBNA-1 peptides are probably also generated by degradation of aberrant translation products in a proteasome-dependant manner (Khanna *et al.*, 1996; Lee *et al.*, 1996; Lautscham *et al.*, 2003). Several papers have now shown that the anti-EBNA-1 $CD8^+$ T-cells are present in most EBV-infected healthy individuals, however, only with more sensitive IFN-γ detection are these CTL detected and not with less sensitive killing assays (Meij *et al.*, 2002; Lee *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004). Given this new information the overall role of EBNA-1 in protection from $CD8^+$ T-cell responses needs to be evaluated. There is a possibility that EBV has evolved a mechanism for allowing limited CTL recognition of infected cells in order to maintain its latency program.

KSHV, like EBV, seems to have evolved a similar mechanism for blocking CTL recognition of its major latency-associated protein, LANA. Like EBNA-1 for EBV, LANA acts in maintenance of the KSHV episome by tethering it to the cellular chromosome (Barbera *et al.*, 2006). Also like EBNA-1, LANA contains long repetitive sequences, that can be broken up into three sections composed of repeats of aspartic acid and glutamic acid, glutamine and glutamic acid, or aspartic acid and glutamine, respectively. These repeat sequences are capable of blocking the processing of CTL epitopes *in cis*, but not *in trans* (Zaldumbide *et al.*, 2006). Further exploration of this function by the Moore group has demonstrated that the block occurs both through synthesis retardation and reduced defective ribosomal product (DRiP) formation and processing (P Moore, personal communication). The overall contribution of this viral impediment to MHC class I antigen presentation on viral immune escape still requires further investigation.

The BCRF1 protein, described earlier in this chapter as a deregulator of the interferon responses, is also able to block MHC class I-dependent CTL responses against viral antigens (Fig. 31.5). This protein, like cellular IL-10, is able to down regulate the transporter associated with antigen processing subunit 1 (TAP1) and a proteasome subunit, low molecular weight protein 2 (LMP2), but not the TAP2 protein (Zeidler *et al.*, 1997). The TAP proteins act to transport peptides from the cytosol into the ER, where they can be loaded onto MHC class I, while LMP2 is a constituent of the proteasome, which degrades antigenic proteins into peptides. After treatment of primary tonsillar B cells with human or viral IL-10 both TAP1 and LMP2 mRNA levels were seen to decrease dramatically (Zeidler *et al.*, 1997). So, EBV has hijacked an immunoregulatory cytokine, which likely plays a role in preventing autoimmune responses, to dramatically decreases the $CD8^+$ CTL antiviral responses (Fig. 31.5).

Evasion of $CD4^+$ T helper cell and B cell responses

The $CD4^+$ T helper (Th) cells are able aid in the recognition and elimination of pathogens in multiple ways. The

Table 31.7. Viral B-cell regulators. The B-cell response is controlled both by signals given directly to the antibody-producing B-cell and signals transmitted to T-helper cells, which in turn aid the B-cell response. Both human γ-herpesviruses encode multiple gene products with the potential to alter the host's humoral response through interfering with both of these aspects of B-cell stimulation. The open reading frame, product and potential function are listed for each, with additional details in the text

	Gene	Product	Function
HHV-8	ORF4	KCP/Kaposica	Accelerates the decay of the C3 convertases and drives production of C3d, which binds complement receptor 2 (CR2) found on B cells
	K5	K5 (MIR-2)	Down regulates ICAM-1 and B7.2 reducing T helper cell activation and reducing the B cell response
EBV	BLLF1a/b	gp350/220	Binds to CR2, might interfere with complement mediated B cell stimulation or aid in viral dissemination
	BZLF1	Zta	Down-regulates MHC class II through CIITA inhibition blocking T helper cell activation

Th1 cells, after recognition of foreign peptides complexed with MHC class II, are able to activate macrophages, as well as activating B cells to produce certain subclasses of antibodies. The CD Th2 cells, in an analogous way, are able to drive the activation and differentiation of B-cells such that they produce a wide variety of immunoglobulins. The B-cell responses are closely tied to this activation of the $CD4^+$ T helper cells and binding of non-self peptides by MHC class II. On the surface of B cells, the B cell antigen receptor (BCR) can bind to antigens, which are then internalized and degraded into peptides that are loaded onto MHC class II. These complexes are transported to the cell surface, where they can be recognized by antigen-specific Th2 cells causing the T-cell to produce both cell surface and secreted proteins. This T-cell help causes the B cell to proliferate and its progeny to differentiate into antibody-secreting cells. The threshold of this proliferation and antibody production can be significantly lowered if the B cell is additionally stimulated through the B-cell coreceptor made up of CD19, CD21 and CD18. Antibodies produced by the activated, differentiated B-cells can then act to neutralize and clear free virus, as well as drive antibody-dependent cell-mediated cytotoxicity (ADCC) reactions where NK cells can target infected cells through Fc receptors on their surface.

Both KSHV and EBV have mechanisms by which they can possibly block B-cell responses as outlined in Table 31.7 and in Fig. 31.5. One component of the B-cell coreceptor, CD21, is capable of binding to C3d. The KSHV KCP/Kaposica protein drives inactivation of the complement C3 convertase and production of C3d, detailed more fully earlier in this chapter (Spiller *et al.*, 2003). It is unclear whether this aberrant production of C3d is capable of altering B-cell responses. It is also possible that C3d is produced in order to attract $CD21^+$ B-cells, which KSHV can then target for infection. EBV also targets CD21 through its gp350/220 envelope protein. This protein is a constituent of the viral envelope and is capable of binding to CD21 to aid in viral entry. Again, it is unclear what implications this has for anti-viral B cell responses.

Much in the same way that the KSHV K5 (MIR-2) protein was able to block the anti-viral activities of the NK cells, it is also able to inhibit T-helper cell activation (Coscoy and Ganem, 2001). Both ICAM-1 and B7.2 play crucial roles in inducing T-help. The down regulation and destruction of these molecules, therefore, prevents the induction of a vigorous B cell response (Coscoy and Ganem, 2001). The presence of multiple cytokines that enhance the Th2 response, however, might diminish the immune evasion potential of this mechanism.

The EBV BZLF1 protein, earlier introduced as having a role in altering interferon responses, is able to block IFN-γ-induced MHC class II surface expression by inhibiting the CIITA transcription factor (Morrison *et al.*, 2001). This has the effect of shutting down T-helper cell activation, limiting the humoral response. While a similar mechanism has not been described for KSHV, the presence of multiple genes capable of interfering with the actions of IFN-γ leave open the possibility that MHC class II induction and stimulation of T-help is being blocked in a similar manner.

Finally, the GAR region of the EBNA-1 protein of EBV was originally thought to limit the $CD4^+$ T-cell responses. This doesn't, however, seem to be true. Several groups have demonstrated an ability to detect strong EBNA-1 $CD4^+$ Th1 responses in healthy individuals (Munz *et al.*, 2000; Bickham *et al.*, 2001; Leen *et al.*, 2001; Paludan *et al.*, 2002; Voo *et al.*, 2002). These cells are capable of recognizing LCLs, EBV transformed cells and Burkitt's lymphoma cell lines (Munz *et al.*, 2000; Bickham *et al.*, 2001; Paludan *et al.*, 2002; Voo *et al.*, 2002). Thus, the role of EBNA-1 in escape from Th and B-cell responses needs further evaluation.

Conclusions

An understanding of the mechanisms by which these viruses evade the antiviral immune responses is informative on several levels. First, by examining viral inhibition of specific immune responses much can be learned about regulation and functioning of the immune system. Second, virally-associated neoplasms can be viewed as aberrations where the normal balance between control of the virus by the host immune responses and avoidance of those same responses by the virus has been corrupted. By understanding what responses are capable of controlling viral proliferation in the case of the immunocompetent host then more effort can be directed at vaccinating to induce protective responses.

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Part III

Pathogenesis, clinical disease, host response, and epidemiology: alphaherpes viruses

Edited by Ann Arvin and Richard Whitley

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Pathogenesis and disease

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Pathogenesis

The transmission of herpes simplex virus (HSV) infection is dependent upon intimate, personal contact of a susceptible seronegative individual with someone excreting HSV. Virus must come in contact with mucosal surfaces or abraded skin for infection to be initiated. With viral replication at the site of primary infection, either an intact virion or, more simply, the capsid is transported retrograde by neurons to the dorsal root ganglia where, after another round of viral replication, latency is established (Fig. 32.1(a), left panel). The more severe the primary infection, as reflected by the size, number, and extent of lesions, the more likely it is that recurrences will ensue. Although replication sometimes leads to disease and, infrequently, results in life-threatening infection (e.g., encephalitis), the host-virus interaction leading to latency predominates. After latency is established, a proper stimulus causes reactivation; virus becomes evident at mucocutaneous sites, appearing as skin vesicles or mucosal ulcers (Fig. 32.1(b), right panel).

Infection with HSV-1 generally occurs in the oropharyngeal mucosa. The trigeminal ganglion becomes colonized and harbors latent virus. However, it has been increasingly common to detect evidence of HSV-1 in the genital tract, usually the consequence of oral-genital sex. When such occurs, recurrences of HSV-1 in the genital tract are uncommon. Acquisition of HSV-2 infection is usually the consequence of transmission by genital contact. Virus replicates in the genital, perigenital or anal skin sites with seeding of the sacral ganglia (Fig. 32.2). As is the case of HSV-1's ability to infect the genital tract, HSV-2 can infect the mouth. Recurrences at this site are uncommon.

Operative definitions of the nature of the infection are of pathogenic relevance. Susceptible individuals (namely, those without pre-existing HSV antibodies) develop primary infection after the first exposure to either HSV-1 or HSV-2. A recurrence of HSV is known as "recurrent infection." Initial infection is when an individual with pre-existing antibodies to one type of HSV (namely, HSV-1 or HSV-2) can experience a first infection with the opposite virus type (namely, HSV-2 or HSV-1, respectively). Primary infection has, more recently, been labeled first-episode disease because some individuals present with what appears to be a clinically severe primary infection but have pre-existing antibodies to the causative type. This observation indicates that individuals may have a well-established latent infection before the first episode of clinically evident disease occurs.

Reinfection with a different strain of HSV can occur, albeit extremely uncommon in the normal host and is called exogenous reinfection. Cleavage of DNA from an HSV isolate by restriction endonuclease enzymes yields a characteristic pattern of subgenomic products. Analyses of numerous HSV-1 and HSV-2 isolates from a variety of clinical situations and widely divergent geographic area demonstrates that epidemiologically unrelated strains yield distinct HSV DNA fragment patterns. In contrast, fragments of HSV DNA derived from the same individual obtained years apart, from monogamous sexual partners, or following short and long passages *in vitro*, have identical fragments after restriction endonuclease cleavage (Buchman *et al.*, 1978). Utilizing endonuclease technology, exogenous reinfection is exceedingly low in the immune competent host.

Unique biologic properties of HSV that influence pathogenesis

HSV-1 and HSV-2 exhibit two unique biologic properties that influence pathogenesis and subsequent human disease. Both viruses have the capacity to invade and replicate

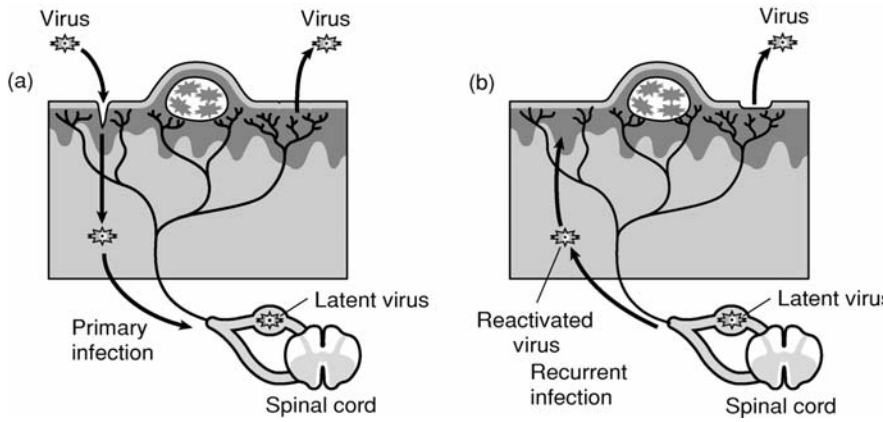

Fig. 32.1. (a) Primary infection. (b) Recurrent infection.

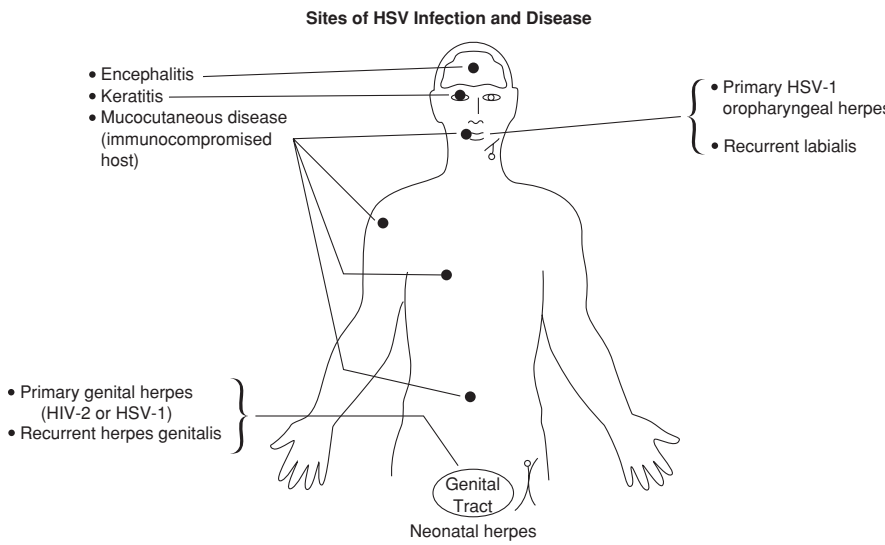

Fig. 32.2. Sites of HSV infection and disease (Whitley, 2001).

in the CNS and the capacity to establish a latent infection in dorsal root ganglia (Roizman and Pellett, 2001).

The term, neurovirulence, encompasses both neuroinvasiveness from peripheral sites and replication in neuronal cells. When paired isolates (brain and lip) from patients with HSV encephalitis are evaluated by PFU/LD_{50} ratios following direct intracerebral inoculation in mice, the encephalitis isolates have lower PFU/LD_{50} ratios than isolates from lip lesions. Neurovirulence appears to be the function of numerous genes (Roizman and Knipe, 2001). In fact, deletion of virtually any of the genes dispensable for viral replication in cell culture reduces the capacity of the virus to invade and replicate in CNS. Mutations affecting neuroinvasiveness have also been mapped in genes encoding glycoproteins. Access to neuronal cells from usual portals of entry into the body requires postsynaptic transmission of virus and, therefore, a particularly vigorous capacity to multiply and to direct the virions to appropriate membranes. In addition, since neuronal cells are terminally differentiated and do not make cellular DNA, they lack the precursors for viral DNA synthesis that are also encoded by the viral genes dispensable for growth in cell culture. Of particular interest, however, is the role of $\gamma_1$34.5 gene in neurovirulence (Chou *et al.*, 1990; Chou and Roizman, 1986; Hesselgesser and Horuk, 1999; Whitley *et al.*, 1993). Although $\gamma_1$34.5 deletion mutants multiply well in a variety of cells in culture, they are among the most avirulent mutants identified to date in vivo.

Latency has been recognized biologically since the beginning of the century (Baringer and Swoveland, 1973; Bastian *et al.*, 1972; Stevens and Cook, 1971) and has been extensively reviewed (Roizman and Knipe, 2001; Nahmias and Roizman, 1973; Roizman and Sears, 1987). The molecular basis for latency is addressed in Chapter 33. Following entry, both HSV-1 and HSV-2 infect nerve endings and translocate by retrograde transport to the nuclei of sensory ganglia. The virus multiplies in a small number of sensory neurons, which are ultimately destroyed. In the vast majority of the infected neurons, the viral genome remains for the entire life of the individual in an episomal state. In a fraction of individuals, the virus reactivates and is moved by anterograde transport to a site at or near the portal of entry. Reactivations occur following a variety of local or systemic stimuli.

Patients treated for trigeminal neuralgia by sectioning a branch of the trigeminal nerve develop herpetic lesions along the innervated areas of the sectioned branch (Carton and Kilbourne, 1952; Cushing, 1905; Goodpasture, 1929; Pazin *et al.*, 1978). Reactivation of latent virus appears dependent upon an intact anterior nerve route and peripheral nerve pathways. Latent virus can be retrieved from the trigeminal, sacral, and vagal ganglia of humans either unilaterally or bilaterally (Bastian *et al.*, 1972). The recovery of virus by in vitro cultivation of trigeminal ganglia helps explain the observation of vesicles that recur at the same site in humans, usually the vermilion border of the lip. Recurrences occur in the presence of both cell-mediated and humoral immunity. Recurrences are spontaneous, but there is an association with physical or emotional stress, fever, and exposure to ultraviolet light, tissue damage, and immune suppression. Recurrent herpes labialis is three times more frequent in febrile patients than in non-febrile controls (Baringer and Swoveland, 1973; Roizman and Sears, 1987; Selling and Kibrick, 1964).

Little is known regarding the mechanisms by which the virus establishes and maintains a latent state or is reactivated. There are in fact disagreements on the fate of neurons in which latent virus became reactivated. The relevant issues may be summarized as follows.

1. Sensory neurons harboring virus contain nuclear transcripts arising from approximately 8.5 kbp of the sequences flanking the U_L sequence. These transcripts are known as the latency associated transcripts or LATs. A shorter region is more abundantly represented in the nuclei. The RNA transcribed from this region forms two populations 2 kbp and 1.5 kbp, respectively, and represents stable introns of an unknown, and relatively unstable transcript. The abundant 2.5 and 1.5 kbp RNA play no role in the establishment or maintenance of the latent state although they may play a role in reactivation. These LATs may have an apoptotic function, which might explain the higher efficiency of reactivation of viruses expressing LATs.

   The source of genetic functions required for the establishment or maintenance of the latent state remains unknown. All of the deletion mutants tested to date establish latency but not all reactivate. Whereas establishment or maintenance of latency are functions expressed by dorsal root neurons, the activation of viral gene expression that leads to viral replication does require a full complement of viral gene.
2. Usually, replication of HSV-1 and HSV-2 destroys the infected cell, but reactivation of latent virus may not destroy neurons harboring the virus. This suggestion is based on the observation that patients do not suffer from local anesthesia at the site of frequent, multiple recurrences. An alternative explanation is that nerve endings from adjacent tissues innervated by other neurons extend into the site of the healed lesion (Roizman and Knipe, 2001; Roizman and Sears, 1987).

Pathology

The histopathologic characteristics of a primary or recurrent HSV (Fig. 32.3) reflect viral-mediated cellular death and associated inflammatory response. Viral infection induces ballooning of cells with condensed chromatic within the nuclei of cells, followed by nuclear degeneration, generally within parabasal and intermediate cells of the epithelium. Cells lose intact plasma membranes and form multinucleated giant cells. With cell lysis, a clear (referred to as vesicular) fluid containing large quantities of virus appears between the epidermis and dermal layer. The vesicular fluid contains cell debris, inflammatory cells, and often multinucleated giant cells. In dermal substructures there is an intense inflammatory response, usually in the corium of the skin, more so with primary infection than with recurrent infection. With healing, the vesicular fluid becomes pustular with the recruitment of inflammatory cells and scabs. Scarring is uncommon. When mucous membranes are involved, vesicles are replaced by shallow ulcers.

Pathology of central nervous system disease

HSE results in acute inflammation, congestion and/or hemorrhage, most prominently in the temporal lobes and usually asymmetrically in adult (Boos and Esiri, 1986) and

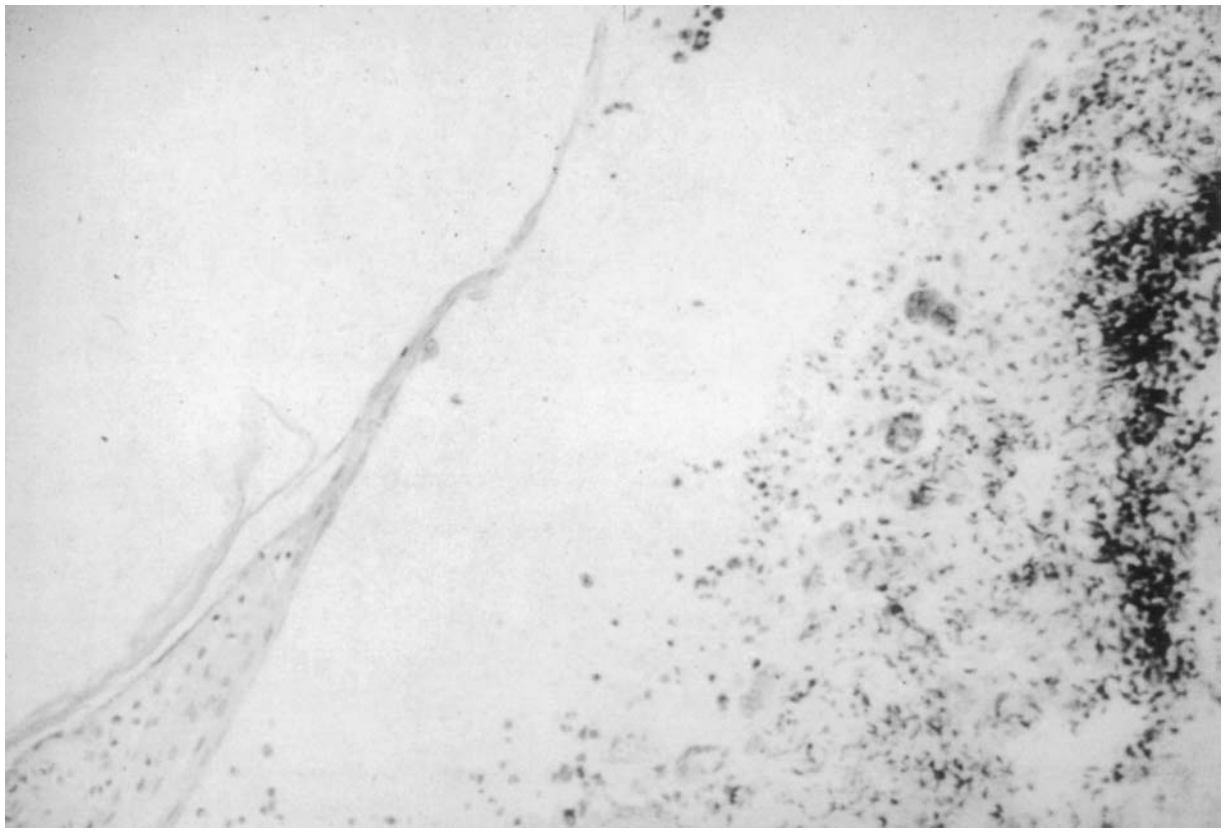

Fig. 32.3. Histopathology of herpes simplex virus infection (Whitley, 2001).

more diffusely in the newborn. Adjacent limbic areas show involvement as well. The meninges overlying the temporal lobes may appear clouded or congested. After approximately 2 weeks, these changes proceed to frank necrosis and liquefication, as shown in Fig. 30.4. Microscopically, involvement extends beyond areas that appear grossly abnormal. At the earliest stage, the histologic changes are not dramatic and may be non-specific. Congestion of capillaries and other small vessels in the cortex and subcortical white matter is evident; other changes are also evident, including petechiae. Vascular changes that have been reported in the area of infection include areas of hemorrhagic necrosis and perivascular cuffing (Fig. 32.5(a), (b)). The perivascular cuffing becomes prominent in the second and third weeks of infection. Glial nodules are common after the second week (Boos and Kim, 1984; Kapur *et al.*, 1994). The microscopic appearance becomes dominated by evidence of necrosis and, eventually, inflammation; the latter is characterized by a diffuse perivascular subarachnoid mononuclear cell infiltrate, gliosis, and satellitosis-neuronophagia (Boos and Esiri, 1986; Garcia *et al.*, 1984). In such cases, widespread aras of hemorrhagic necrosis, mirroring the area of infection, become most prominent. Oligodendrycytic involvement and bliosis (as well as astrocytosis) are common, but these changes develop very late in the disease. Although found in only approximately 50% of patients, the presence of intranuclear inclusions supports the diagnosis of viral infection, and these inclusions are most often visible in the first week of infection. Intranuclear inclusions (Cowdry type A inclusions) are characterized by an eosinophilic homogeneous appearance and are often surrounded by a clear, unstained zone beyond which lies a rim of marginated chromatin, as shown in Fig. 32.6.

Impact of host response to infection on disease

The pathogenesis of HSV infections is influenced by both specific and non-specific host defense mechanisms (Lopez *et al.*, 1993). With the appearance of non-specific inflammatory changes, paralleling a peak in viral replication, specific host responses can be quantitated but vary from one

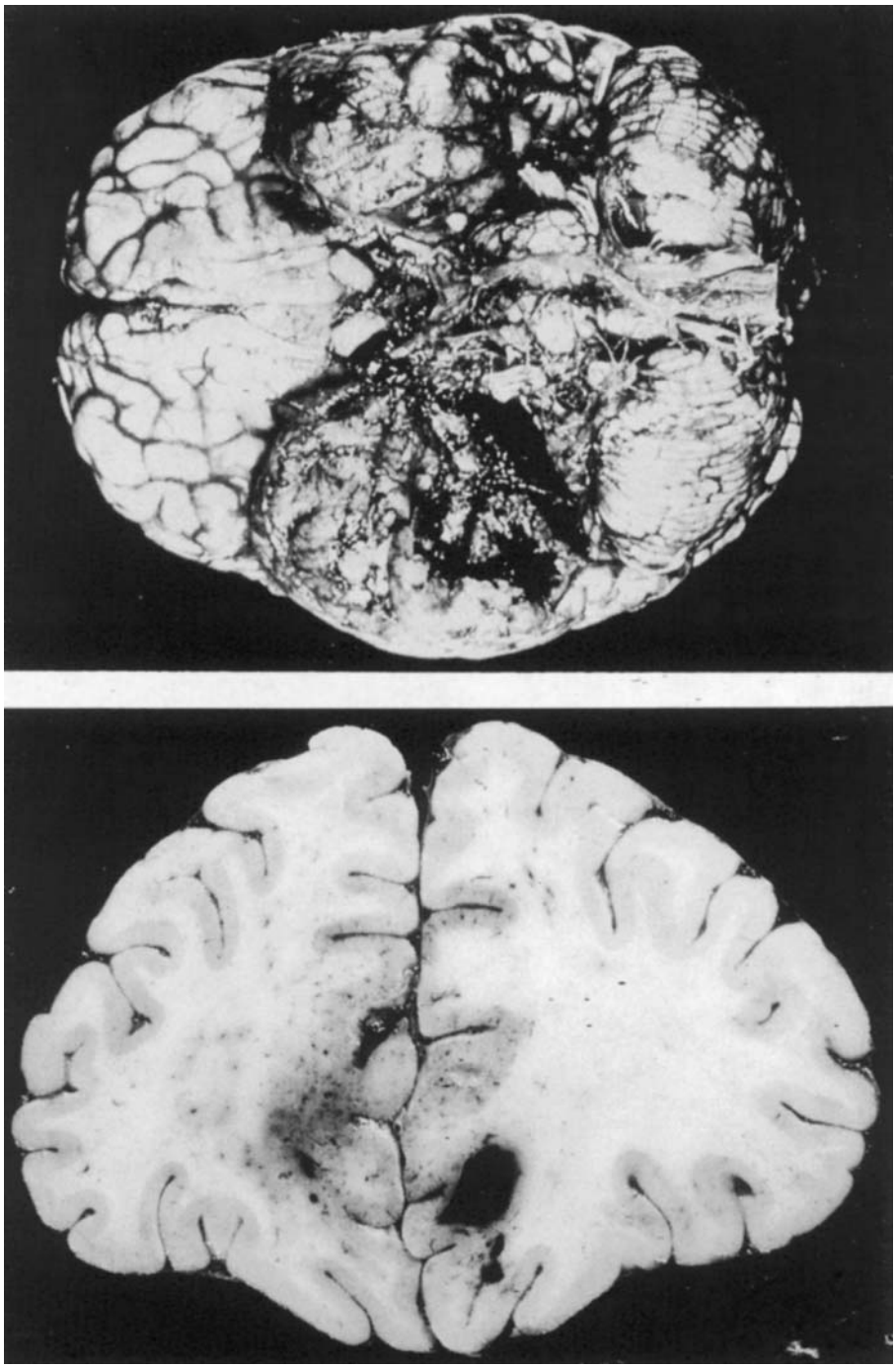

Fig. 32.4. Gross pathologic findings in HSE, illustrating hemorrhagic necrosis of the inferior medial portion of the temporal lobe (Whitley, 2001).

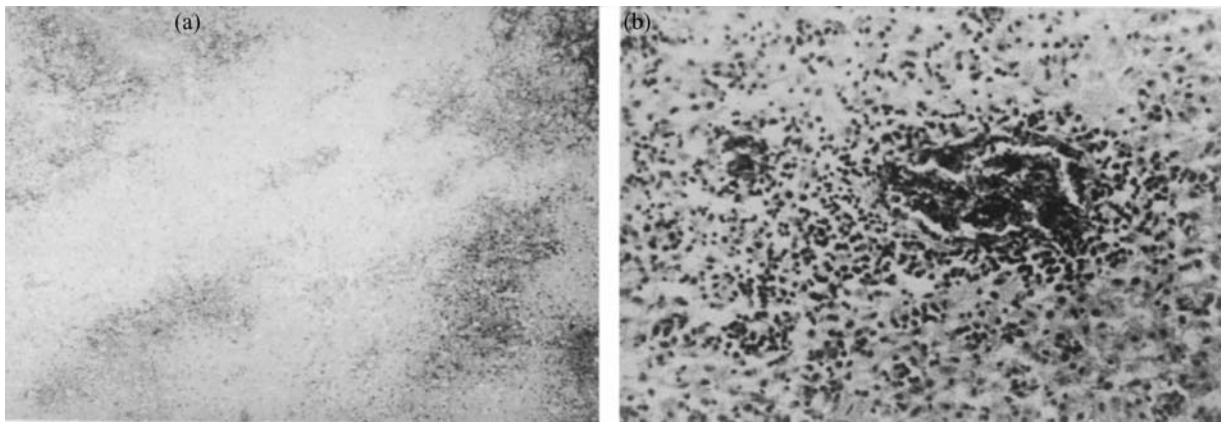

Fig. 32.5. (a) Hemorrhagic necrosis on microscopic examination (Whitley, 2001). (b) Perivascular cuffing on histopathologic examination of a patient with HSE (Whitley, 2004).

animal system to the next. In the mouse, delayed-type hypersensitivity responses are identified within 4–6 days after disease onset, followed by a cytotoxic T-cell response and by the appearance of both IgM- and IgG-specific antibodies. Host responses in humans are delayed, developing approximately 7–10 days later. Immunodepletion studies have identified the importance of cytotoxic T-cells (CTLs) in resolving cutaneous disease. Adoptive transfer of $CD8^+$ or HSV-immune $CD4^+$ T cells also reduces viral replication or protection from challenge.

T-cell lymphocyte subsets have been examined for host susceptibility to infection, including those cells responsible either for H2-restricted cytotoxicity or for in vitro or adoptive transfer of delayed-type hypersensitivity (Kohl *et al.*, 1989). These latter cells have a requirement for both the IA and H2 K/D regions (Nash *et al.*, 1981). Studies utilizing a specific infected cell polypeptide product (ICP4) have identified its requirement for mediation of T-cells (Martin *et al.*, 1988). Prior immune responses to HSV-1 infection have a protective effect on the acquisition of HSV-2 infection (Mertz *et al.*, 1992). Polyclonal antibody therapy will decrease mortality rates in the newborn mouse (Brown *et al.*, 1991). In addition, administration of these antibodies can limit progression of both neurologic and ocular disease. Protection can be achieved with monoclonal antibodies to specific viral polypeptides, especially the envelope glycoproteins. Such results have been accomplished with both neutralizing and non-neutralizing antibodies. Antibody-dependent cell-mediated cellular cytoxic host responses also correlate with improved clinical outcome, as will be noted below for neonatal HSV infections.

Numerous reports have incriminated or refuted HLA associations with human HSV infections. For recurrent fever blisters, these studies have included HLA-A1, HLA-A2, HLA-A9, HLA-BW16, and HLA-CW2. Recurrent ocular HSV infections have been associated with HLA-A1, HLA-A2, HLA-A9, and HLA-DR3. These conflicting associations can be faulted by population selection bias.

Humoral immune responses of humans parallel those following systemic infection of mice and rabbits. IgM antibodies appear transiently and are followed by IgG and IgA antibodies, which persist over time. Neutralizing and antibody-dependent cellular cytotoxic antibodies generally appear 2–6 weeks after infection and persist for the lifetime of the host. Immunoblot and immunoprecipitation antibody responses have defined host response to infected cell polypeptides and correlated these responses with the development of neutralizing antibodies (Bernstein *et al.*, 1985; Eberle *et al.*, 1981). After the onset of infection, antibodies appear which are directed against gD, gB, ICP-4, gE, gG-1 or gG-2, and gC. Both IgM and IgG antibodies can be demonstrated, depending upon the time of assessment.

Lymphocyte blastogenesis responses develop within 4–6 weeks after the onset of infection and sometimes as early as 2 weeks (Corey *et al.*, 1978; Russell, 1974; Sullender *et al.*, 1987). With recurrences, boosts in blastogenic responses can be defined promptly; however, these responses, as after primary infection, decrease with time. Non-specific blastogenic responses do not correlate with a history of recurrences.

Host response of the newborn to HSV differs from that of older individuals. Impairment of host defense mechanisms contributes to the increased severity of some infectious agents in the fetus and the newborn. Factors which must be considered in defining host response of the newborn include the mode of transmission of the agent (viremia vs mucocutaneous infection without blood-borne spread), and time of acquisition of infection.

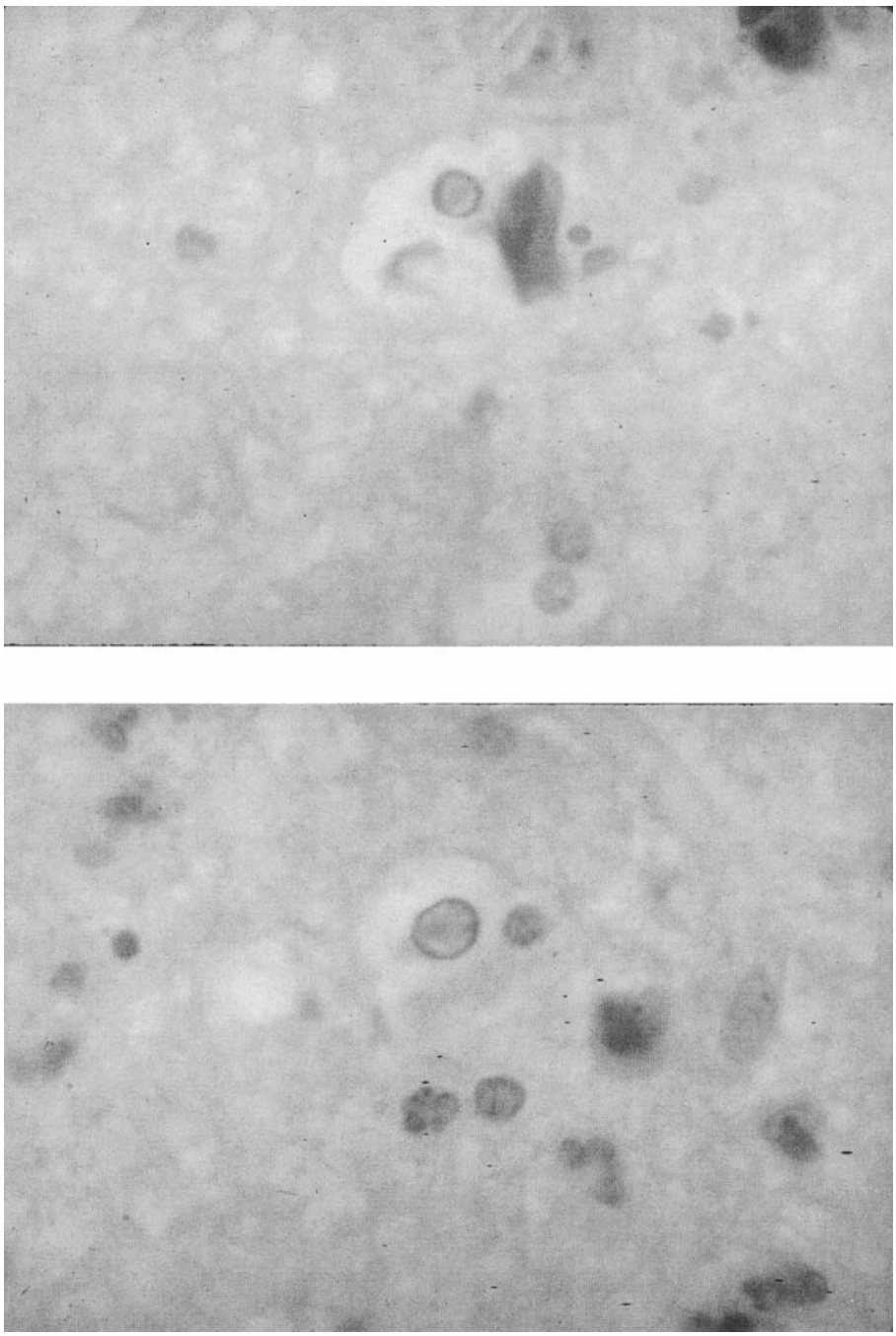

Fig. 32.6. Intranuclear inclusions (Whitley, 2004).

Humoral immunity does not prevent either recurrences or exogenous reinfection. Thus, it is not surprising that transplacentally acquired antibodies from the mother are not totally protective against newborn infection (Kohl *et al.*, 1989; Sullender *et al.*, 1987). The quantity of neutralizing antibodies is higher in those newborns who do not develop infection when exposed to HSV at delivery (Prober *et al.*, 1987). Transplacentally acquired neutralizing antibodies either prevent or ameliorate infection in exposed newborns, as do antibody-dependent cell-mediated cytotoxic antibodies (Prober *et al.*, 1987). Nevertheless, the presence of antibodies at the time of disease presentation does not

necessarily influence the subsequent outcome (Whitley *et al.*, 1988; Whitley *et al.*, 1980).

Infected newborns produce IgM antibodies (as detected by immunofluorescence) specific for HSV within the first 3 weeks of infection and increase rapidly during the first 2–3 months, being detectable for as long as 1 year after infection. The most reactive immunodeterminants are the surface viral glycoproteins, particularly gB and gD (Sullender *et al.*, 1987).

Newborns infected by HSV have a delayed T-lymphocyte proliferative response as compared to that of older individuals (Sullender *et al.*, 1987). Most infants have no detectable T-lymphocyte responses to HSV 2–4 weeks after the onset of clinical symptoms (Sullender *et al.*, 1987; Rasmussen and Merigan, 1978). These delayed responses may be associated with disease progression (Sullender *et al.*, 1987).

Infected newborns have decreased α-interferon production in response to HSV antigen as compared to adults with primary HSV infection (Sullender *et al.*, 1987). Lymphocytes from infected babies also have decreased responses to α-interferon generation (Sullender *et al.*, 1987).

Disease consequences

Most individuals who have prior serologic evidence of infection with HSV-1 and/or HSV-2 do not recognize that they have been infected (Whitley and Roizman, 2001). Therefore, most infections are asymptomatic or associated with non-specific signs and symptoms. However, when symptoms do occur, they tend to be more severe with primary compared with recurrent infections. Also, whether accompanied by symptoms or not, viral excretion during primary infection is more prolonged than shedding during recurrent infection.

The most common sites of HSV infection include the skin and mucosal surfaces. HSV-1 and HSV-2 infections tend to be transmitted by different routes and infect different areas of the body but signs and symptoms of infection with either virus are similar (Whitley and Roizman, 2001). In general, infections caused by HSV-1 occur above the waist and those caused by HSV-2 occur below the waist. However, over the last several decades considerable overlap in site of infection has evolved.

Orolabial infection

Primary infection

The oropharynx is the most common site of infection caused by HSV-1. Although most primary orolabial infections appear to be mild or asymptomatic, some young children develop extensive orolabial lesions accompanied by systemic symptoms. A typical course of severe infection includes, high fever, irritability, tender submandibular lymphadenopathy, and a widespread mucocutaneous eruption. Vesiculo-ulcerative lesions involve the palate, gingiva, tongue, lip, and perioral area (Kuzushima *et al.*, 1991). Dehydration, due to impaired eating and drinking, is the most common reason for hospital admission (Cesario *et al.*, 1969). Symptomatic primary infection may evolve over 2 to 3 weeks.

Primary HSV infection in older children and adults can present as pharyngitis. HSV has been isolated from the posterior pharynx of up to 24% of college students with symptoms of pharyngitis, including pharyngeal erythema, exudative or ulcerative lesions on the posterior pharynx and tonsils, enlarged cervical lymph nodes, and fever (McMillan *et al.*, 1993).

Reactivation

Reactivation of HSV from the trigeminal ganglia often is asymptomatic; silent excretion of virus by healthy previously infected individuals occurs on about 1% of days for children and 5% to 10% of days for adults (Scott *et al.*, 1997). In some individuals, viral reactivation, with or without associated symptoms occurs in association with fever, exposure to ultraviolet radiation or wind, non-specific stresses, manipulation of the trigeminal nerve root, or dental extraction (Openshaw and Bennett, 1982). It is estimated that 20 to 40% of adults experience recurrent herpes labialis (Bader *et al.*, 1978; Lowhagen *et al.*, 2002).

The outer edge of the vermilion border is the most common site of reactivation; on average three to five lesions are present. The lesions usually begin as vesicles, evolve into pustules or ulcers after 1 to 2 days, and heal within 8 to 10 days. Prodromal symptoms including burning, itching, or tingling may precede the outbreak by several hours and pain, when evident, is maximum at the onset of eruption, resolving after 4 to 5 days (Spruance *et al.*, 1977).

Genital infection

Primary infection

The majority of primary genital herpes infections occur in the absence of symptoms. When symptoms do occur, they tend to be more severe when infection is caused by HSV-2 than HSV-1 (Whitley, 2001). Systemic symptoms, including headache, fever, myalgia, and backache occur in about 70% of women and 40% of men seeking medical care for primary genital herpes (Corey *et al.*, 1983). These symptoms peak during the first 4 days of infection and abate

over the subsequent 7 to 10 days. Itching and local pain often precede visible lesions by 1 to 2 days. Lesions erupt over 7 to 8 days and evolve from vesicles and pustules to wet ulcers over approximately 10 days; crusting and healing follows over the ensuing 10 days. Common sites for lesions in women are the labia majora, labia minora, mons pubis, vaginal mucosa, and cervix. Lesions in men typically are found on the shaft of the penis. More than 80% of women and 40% of men have dysuria for 7 to 10 days. Tender inguinal adenopathy appears during the second to third week of illness and is generally the last sign to resolve. Complications are more common in women than men and include aseptic meningitis, paraesthesias and dysaesthesias of the legs and perineum, mucocutaneous lesions beyond the genital area, pharyngitis, and visceral dissemination. Perianal infection and proctitis are common in men who have sex with men.

Reactivation

Most recurrences of genital herpes are asymptomatic and, on any given day, symptomless shedding occurs in approximately 3 to 5% of women previously infected with HSV-2 (Wald *et al.*, 1997). Importantly, when PCR is used to detect evidence of HSV excretion in the genital tract of women known to have genital herpes, infectivity increases by at least fourfold. Thus, these women can be infectious as often as one out of four days.When symptoms do occur, they tend to be mild; constitutional complaints are present in less than 10% of patients and local prodromal symptoms are apparent in less than 50% (Corey *et al.*, 1983). Genital lesions are few in number and localized; they typically evolve from vesicle to healing in 8 to 10 days. The buttock, thighs and perianal mucosa may be unrecognized sites of recurrent infection. It has been suggested that herpes infection be considered in the differential diagnosis of unexplained recurrent itching, burning, blistering, or erythema at any site below the waist (Simmons, 2002). Approximately one-third of patients will not have recurrent infections, one-third will have two recurrences per year, and one third will have more than six recurrences per year (Whitley, 2001). Emotional stress, menses, and sexual intercourse have been some of the factors implicated in precipitating recurrences.

Keratoconjunctivitis

Herpes simplex virus is a major cause of ocular scarring and visual loss (Simmons, 2002). It is estimated that in excess of 300 000 cases of HSV eye infections are diagnosed each year in the United States (Whitley *et al.*, 1998). Beyond the neonatal period, the majority of these infections are caused by HSV-1. Infection may be unilateral or bilateral, beginning with follicular conjunctivitis associated with pain, photophobia, and tearing and followed by chemosis, periorbital edema, and preauricular lymphadenopathy (Pavan-Langston, 1990). Progressive infection may result in sight-threatening corneal ulcers, characterized by pathognomonic branching dendritic lesions. Healing may be slow, requiring more than 1 month. About one-third of individuals develop recurrences during the ensuing 5 years.

Cutaneous infections

HSV can infect virtually any part of the skin or mucosa. One of the most common cutaneous sites for HSV-1 or HSV-2 infection is the pulp or nail bed of the finger. This is referred to as herpetic whitlow and most commonly occurs in medical and dental professionals, in whom it results from digital contamination with genital or oral secretions (Feder and Long, 1983). When a young child develops herpetic whitlow, it may result from autoinoculation during primary oral herpes infection or when an infected adult trims the child's nails by biting (Feder and Long, 1983). The typical clinical course of whitlow involves the initial appearance of discrete vesicular or pustular lesions over the distal phalynx which subsequently coalesce over several days. Pain often is associated with a tingling or burning sensation. Fever, lymphangitis, and tender swelling of local lymph nodes may be present. The diagnosis of herpetic whitlow is most often confused with bacterial cellulitis.

Close contact between abraded skin and oral secretions results in cutaneous infections caused by HSV-1 among participants in certain contact sports including wrestlers (herpes gladiatorum) and rugby players (scrumpox) (Becker *et al.*, 1988; Stacey and Atkins, 2000). In descending order, the most common sites of infection among wrestlers are the head, extremities, and trunk (Belongia *et al.*, 1991). About 40% of infected athletes have associated sore throat and 25% have fever, chills, and headache (Belongia *et al.*, 1991). Herpes infections also can result in severe cutaneous infection when they occur on skin damaged by diaper dermatitis, burns, or atopic dermatitis (Jenson and Shapiro, 1987; Wheeler and Abele, 1966; McMill and Cartotto, 2000). Finally, HSV is the most common precipitating factor for recurrent erythema multiforme (Orton *et al.*, 1984).

Central nervous system infections

Herpes simplex viruses cause a variety of peripheral and CNS illnesses of infectious and post-infectious nature

(Simmons, 2002; Schmutzhard, 2001). HSV-1 is the most common cause of sporadic severe encephalitis in the United States, accounting for an estimated 10 to 20% of all cases (Lakeman *et al.*, 1995). Without treatment, more than 70% of infected patients die and virtually all survivors have severe sequelae (Whitley, 2001). Encephalitis can result from a primary or, more commonly, a reactivated HSV infection.

Patients typically present with altered state of consciousness, bizarre behavior, and focal neurologic findings, referable to the temporal lobe. Typical abnormalities in the cerebrospinal fluid (CSF) of patients with HSV encephalitis include a few hundred white blood cells/mm^3, with a predominance of lymphoid cells (75% to 100%) and an increased number of red blood cells (Koskiniemi *et al.*, 1984). Protein concentration is normal in about one-half of CSF specimens obtained during the first week of illness, but thereafter concentrations as high as 500 to 1200 mg/dl are common (Koskiniemi *et al.*, 1984). Virus rarely is isolated from CSF but the presence of HSV DNA, identified by polymerase chain reaction (PCR), is sensitive and specific for the diagnosis of HSV encephalitis (Lakeman *et al.*, 1995; Tang *et al.*, 1999).

Typical findings on electroencephalography include focal spike and slow-wave abnormalities, with characteristic paroxysmal lateralizing epileptiform discharges. Focal edema associated with hemorrhagic necrosis may be present on neurodiagnostic images; abnormalities tend to be evident earlier on magnetic resonance imaging than computed tomography.

Other neurologic syndromes associated with HSV infection include recurrent aseptic meningitis (Mollaret's meningitis), brainstem encephalitis, ascending myelitis, post infectious encephalomyelitis, a variety of movement disorders and atypical pain syndromes, and temporal lobe epilepsy (Simmons, 2002; Schmutzhard, 2001).

Neonatal infection

Over 90% of neonatal infections caused by HSV are contracted at the time of delivery (intrapartum infection) but about 5% are contracted in utero (congenital infection). Manifestations of congenital infection include skin lesions and scars, chorioretinitis, microcephaly, hydranencephaly, and microphthalmia (Hutto *et al.*, 1987).

Neonates infected perinatally present with a range of manifestations, categorized as localized to the skin eye and mouth (SEM) or the CNS, or as disseminated infection. In a recent cohort of 79 neonates with HSV infection who were enrolled into a clinical study between 1989 and 1997, 13% had SEM, 35% had CNS, and 52% had disseminated infection (Kimberlin *et al.*, 2001).

Neonates with SEM disease usually present during the first 2 weeks of life; occasionally skin lesions are evident in the delivery room. The cutaneous lesions first appear where there has been trauma, such as the site of attachment of fetal scalp electrodes, the margin of the eyes, or over the presenting body part. Initially the lesions appear as macules but they rapidly evolve to vesicles. Outcome of SEM disease is excellent if diagnosis is considered, and antiviral therapy administered, in a timely fashion (Kimberlin *et al.*, 2001).

Neonatal HSV infection involving the CNS usually results in fever and lethargy, first appearing between the second and third weeks of life. The sign most specific for HSV infection is the presence of skin lesions. However, approximately one-third infants with CNS disease due to HSV infection do not have skin lesions at the time of clinical presentation (Kimberlin *et al.*, 2001). A common but not as specific sign of neonatal HSV infection of the CNS is the sudden onset of seizures that tend to be focal and difficult to control. Usual CSF abnormalities include a mononuclear pleocytosis (<100 white blood cells/mm^3), slightly reduced glucose, and modestly to markedly elevated protein concentration. The electroencephalogram typically is diffusely abnormal and magnetic resonance imaging reveals either temporal or diffuse cerebral disease. If untreated, most neonates with CNS infection caused by HSV die and almost all survivors are left severely neurologically impaired.

Signs of disseminated infection caused by HSV may mimic severe bacterial infection with onset during the first week of life. Common clinical manifestations include vascular instability, hepatomegaly, jaundice, bleeding, and respiratory dysfunction. Approximately 60% of patients develop skin lesions during their illness, but lesions may be absent at the onset of symptoms (Kimberlin *et al.*, 2001). Progression of infection is rapid, with death resulting from shock, liver failure with bleeding, respiratory failure, or neurologic compromise.

Infection in compromised hosts

The likelihood of complicated HSV, with attendant substantial morbidity, parallels the degree of compromise of cellular immune function (Rand *et al.*, 1977). The most frequent complication of HSV infections among immunocompromised patients is slowly progressive and chronic mucocutaneous infections, accompanied by extensive tissue damage and necrosis (Whitley *et al.*, 1984; Whitley, 2004). Contiguous mucosal spread resulting in esophageal,

tracheal, pulmonary involvement or visceral dissemination also can occur but fatal infections are not common. Organ transplant recipients, particularly human stem cell transplant recipients, and individuals with HIV/AIDS are at particular risk for both severe and frequently recurrent infections.

Acknowledgment

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Molecular basis of HSV latency and reactivation

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Introduction

Primary infection with HSV-1 or HSV-2 results in productive replication of the virus at the site of infection, following the pattern of gene expression described elsewhere in this volume. During this initial phase, virus enters sensory neurons via their termini and retrograde transport takes the genome to the neuronal nuclei in the sensory ganglia that innervate the infected dermatome. At early times after infection, virus replication occurs in ganglionic neurons but within a few days no virus can be detected. The genome, however, persists in neurons in a latent state from which it reactivates periodically to resume replication and produce infectious virus. This reactivation event may be "spontaneous" but is generally thought to be provoked by stress stimuli that act on the neuron, or at a peripheral site innervated by the infected ganglion, or systemically. Three phases of latency are recognized. Establishment occurs during the period following primary infection, and although virus replication can be detected in a proportion of neurons during this phase, the initiation and normal progression of productive infection and cell death is arrested in those neurons destined to become latently infected. Unravelling the way in which the seemingly inexorable progression of the gene expression program is blocked constitutes a major challenge for the molecular virologist. The maintenance phase of latency is characterized by the lifelong retention of the HSV genome in a silent state, characterized by repression of all viral lytic genes. One region, encoding the latency-associated transcripts (LATs), remains active during latency. Questions relating to the maintenance of the latent state focus on the structure of the genome, the mechanisms that silence it, and the specific properties of the LAT transcription unit that enable it to remain active. During the reactivation phase the silent genome responds to cellular signals that provoke the resumption of viral gene expression. The molecular basis for this dramatic functional reversal is poorly understood and is the subject of considerable research effort. In view of the specific association of LAT with the latent state, questions concerning the role of this transcript pervade all aspects of latency.

Model systems to study latency

Animal systems provide the most relevant means of studying HSV latency. In mice, latency can be established efficiently and relatively reproducibly after inoculation with HSV-1 in the cornea, ear, footpad, or at other peripheral sites. The virus replicates at the site of inoculation, and as a consequence the exact dose applied to nerve termini is undefined and changing over the first few days. These factors complicate quantitative aspects of latency studies. Reactivation is difficult to achieve in mice and most investigations have relied on explantation of ganglia, with subsequent culture in the laboratory, to recover virus (Stevens and Cook, 1971). An in vivo protocol has been developed in which reactivation is achieved by exposure of mice to transient hyperthermia (Sawtell and Thompson, 1992b). While this method is inefficient in terms of the number of reactivation events per animal, it represents the most relevant mouse model currently available for paralleling latency in humans. In the rabbit, inoculation of the cornea results in latency that, for certain strains of HSV-1 such as McKrea, is characterized by long-term periodic virus shedding, described as spontaneous reactivation. Production of virus can be enhanced by procedures such as iontophoresis of epinephrine into the eye. For HSV-2, inoculation into the guinea pig vagina results in latency in which virus is periodically shed to form lesions that can be scored. Therefore, the rabbit and guinea pig provide reasonable models for

HSV-1 and HSV-2, respectively, in humans but they are more difficult and expensive to use than the mouse. All animal systems entail inoculation with relatively high virus doses, often just short of fatal, and it is by no means clear how this relates to natural infection. A further problem is the considerable variation in the efficiency of establishment of latency and reactivation between strains of HSV, a factor that frequently causes confusion when comparisons are made between the results from different laboratories.

As a more tractable system, infection of cultured neurons has been investigated (Wilcox and Johnson, 1988; Arthur *et al.*, 2001). These cells are susceptible to lytic infection with HSV, but if measures are taken to prevent virus replication, long-term retention of the viral genome can be achieved. LAT can be detected in a proportion of neurons, but all other viral genes are repressed. Production of virus can be induced by removal of nerve growth factor (NGF), inhibition of histone deacetylases, or various treatments that activate signal transduction pathways (Smith *et al.*, 1992). Alternatively, cultures may be made from ganglia dissected from mice harboring latent HSV; in this case, heat shock or treatment with dexamethasone are the most effective reactivation stimuli (Halford *et al.*, 1996).

The final type of model involves the infection of standard tissue culture cells, usually human fibroblasts, with HSV-1 mutants that are impaired for immediate early (IE) gene expression and thus do not kill cells (Preston and Nicholl, 1997; Samaniego *et al.*, 1998). The viral genome is retained in a quiescent state in which all gene expression, including that of LAT, is repressed. The only known way of reactivating quiescent virus is to provide the HSV IE protein ICP0 by superinfection of cultures. Fibroblast systems may mimic some, but certainly not all, aspects of latency.

The latent genome

Latent HSV DNA does not contain detectable termini and almost certainly exists as a circular episome, in contrast to the linear state in the virus particle (Rock and Fraser, 1983; Efstathiou *et al.*, 1986). Quiescent genomes stably retained in fibroblasts are also circular (Jamieson *et al.*, 1995; Jackson and DeLuca, 2003). Various methods of quantifying viral DNA load revealed that latently infected neurons must contain, on average, many more than one HSV genome copy per infected cell. This conclusion has been verified by the use of "contextual analysis" (CXA), in which individual neurons or small groups of cells are separated and analyzed by polymerase chain reaction (PCR) (Sawtell, 1997). The latent viral genome copy number varied generally between 1 and 100, but a small proportion of neurons contained more than 1000 viral DNA molecules per cell. Likely, the retention of such high copy numbers has an influence on neuronal physiology, and recent studies have shown that latently infected ganglia contain increased levels of certain cellular gene products (Kramer *et al.*, 2003). Furthermore, analysis of latent DNA at a gross level is skewed towards the few neurons containing thousands of viral genomes. Surprisingly, for reasons that are not understood, latent viral DNA cannot be detected by *in situ* hybridization (ISH), therefore *in situ* PCR has been applied to investigate the number of neurons that harbor HSV genomes. This approach shows that many more cells contain DNA than are detected by ISH for LAT; thus, LAT is not an unambiguous marker for latent HSV. Laser capture microdissection, in which individual neurons are excised and analyzed by PCR, confirmed that viral genomes can be isolated from LAT-negative (LAT-) neurons and essentially agreed with the quantification from CXA (Chen *et al.*, 2002). The conclusion that there is a population of latently infected neurons that does not express LAT may depend on the sensitivity of ISH, since a study using *in situ* RT-PCR suggested that LAT was present in all HSV DNA-containing neurons, albeit at low concentration in many (Ramakrishnan *et al.*, 1996).

In cells, silencing of large gene blocks occurs at the level of chromatin structure, and it is therefore suspected that an organization of this type applies to the latent viral genome. One study has addressed this issue and found that all regions of HSV DNA examined, including the LAT region, exhibit a regular nucleosomal pattern in mouse brain stem (Deshmane and Fraser, 1989). Interpretation of this result is complicated by the fact that reactivation from brain stem is inefficient and, unfortunately, it was not possible to obtain sufficient material from trigeminal ganglia for similar analyses. More recently, the application of chromatin immunoprecipitation (ChiP) assays has demonstrated the importance of histone modifications in the maintenance of latency. It is well established that post-translational modification of the amino terminal tails of histones is involved in the regulation of transcription. Thus hyperacetylation of histones is generally associated with an "open chromatin" conformation and transcriptional activity, whilst histone hypoacetylation is associated with condensed chromatin and gene silencing. Recent work on HSV-1 suggests that chromatinization of the viral genome and certain accompanying histone modifications offer a means to regulate virus gene expression during lytic infection (Herrera and Triezenberg, 2004; Kent *et al.*, 2004). In the context of latency it is of particular significance that ChiP assays have shown the LAT promoter to be enriched with acetylated

histone H3 whilst representative lytic cycle promoters exhibit a decreased association with acetylated histones (Kubat *et al.*, 2004a,b). The demonstration that enrichment of acetylated histones on the ICP0 promoter following the application of a reactivation stimulus by ganglionic explantation strongly supports the view that genome derepression is linked to the acetylation status of histones positioned on lytic cycle promoters (Amelio *et al.*, 2006). Furthermore, it has been shown that a LAT- mutant exhibits enrichment of histone modifications associated with transcriptional activation during latency, suggesting that LAT-encoded functions facilitate maintenance of a repressed chromatinized genome (Wang *et al.*, 2005). Since HSV replication in ganglia precedes latency, it has long been suspected that some viral genomes are derived from residual replication intermediates rather than from virions delivered from the periphery. During infection with TK-mutants, which replicate at the site of inoculation but not in neurons, high copy number retention of TK-virus genomes is possible and therefore some neurons can receive hundreds of viruses from the periphery (Thompson and Sawtell, 2000). In general, however, TK-mutants deposit less latent DNA than wild type virus. However, depending on the site of inoculation, TK-mutants replicate less efficiently peripherally. Thus, apparently normal latency can be established without viral replication in neurons.

The latency-associated transcripts

The only transcripts detectable during latency are the LATs, which map to the viral repeats flanking U_L (Fig. 33.1). These have been detected in latently infected neuronal tissues from experimentally infected animals and following natural infection in humans (Stevens *et al.*, 1987). Similar transcripts are synthesized during latent infection by HSV-2 and other alphaherpesviruses such as bovine herpesvirus-1 (BHV-1) and pseudorabies virus.

Structure of LATs

In HSV-1, the LATs comprise a series of colinear predominantly nuclear transcripts. They consist of a highly abundant non-polyadenylated major species of 2.0 kb that is derived by splicing from a less abundant precursor RNA termed minor (m) LAT. The mLAT is transcribed antisense to the ICP0 gene and extends to a polyadenylation signal in the short repeat region. Based on the sequence analysis of HSV-1 strain 17, in LAT spans nucleotides 118 801 to 127 143 and consists of a primary transcript of 8.3 kb. Current evidence supports the view that the 2.0 kb major LAT is an unusually stable intron which is present to at least 40 000 copies per cell. The stability of this RNA is a consequence of inefficient debranching of the intron, due to the presence of a unique non-consensus guanosine branchpoint resulting in persistence of major LAT as a lariat. Further splicing of the 2.0 kb major LAT RNA occurs within neurons to produce an additional stable RNA species of 1.5 kb, which is also considered to accumulate as a stable lariat (Zabolotny *et al.*, 1997). A less complex pattern of transcription is observed during lytic infection of cells in culture. In this setting, synthesis of the 2.0 kb LAT can be detected late in infection but there is a notable absence of the 1.5 kb major LAT species. Furthermore, a fully processed transcript composed of the spliced exons of the primary transcript has not been detected during productive or latent infection, presumably reflecting the rapid degradation of this RNA species. ISH studies of latently infected sensory neurons have shown that major LATs have a diffuse nuclear localization pattern whereas mLATs are localized within discrete nuclear foci that may represent sites of accumulation or synthesis (Arthur *et al.*, 1993). In contrast, during productive infection of cells in culture the 2 kb LAT intron is also found in the cytoplasm and associates with both ribosomal and splicing complexes in infected cells (Ahmed and Fraser, 2001). More recently it has been shown that herpesviruses, including HSV-1, encode micro (mi) RNAs (Pfeffer *et al.*, 2005; Cui *et al.*, 2006). Interestingly, a single miRNA generated from the exon 1 region of LATs has been shown to exert an anti-apoptotic effect by targeting transforming growth factor (TGF) beta and SMAD3 expression (Gupta *et al.*, 2006).

The LAT promoter

Analyses of the HSV-1 DNA sequence upstream from the minor LAT transcription start site identified a TATA box (nt 118 647), a CAAT box (nt 118 647), two CREB binding sites, and SP1 binding sites, making this a candidate LAT promoter element (LAP1). To define the role played by LAP1 in LAT synthesis, a small fragment including the TATA box was deleted (Dobson *et al.*, 1989). Although such a virus could establish latency, no LATs were produced. In addition when the rabbit beta-globin gene was inserted downstream of the TATA box, beta-globin specific RNA, but no major LATs, were transcribed in latently infected neurons. These data are consistent with latent phase transcription initiating from LAP1 to produce the large mLAT species, which is subsequently processed to generate the stable major LAT species. Considerable effort has gone into studying the activity of LAP1 in transient assays. These studies have revealed that this promoter has a high basal activity in

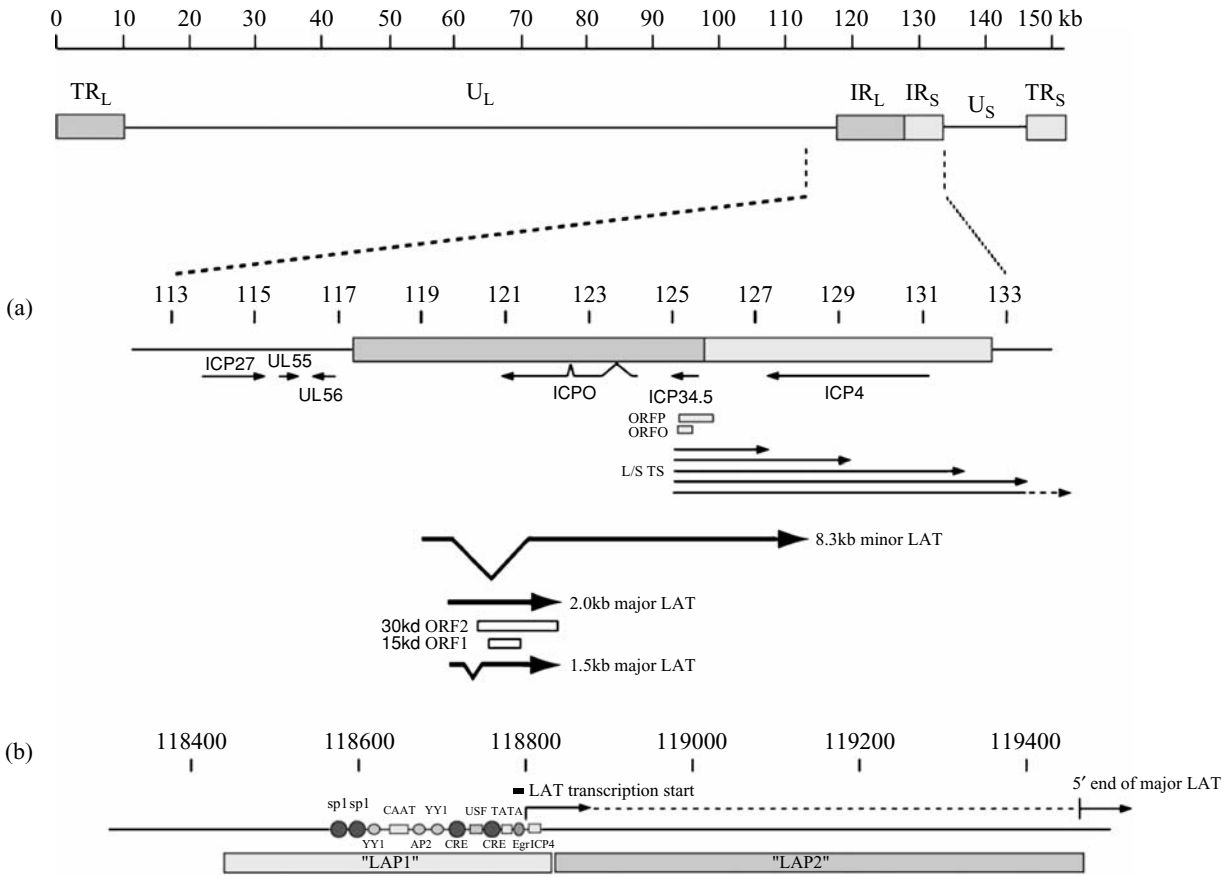

Fig. 33.1. Location and organization of HSV-1 LATs. The IR_L/IR_S region of the genome is expanded in part (a). In addition to the LATs, the positions of lytic cycle genes and a set of transcripts known as L/STs, which specify ORFs O and P, are shown. The functions of L/STs are unclear. The LAP1 and LAP2 promoters are depicted in (b).

a variety of non-neuronal cell types and shows enhanced activity in cells of neuronal origin, an observation consistent with the identification of neuron specific transcription factors which bind to upstream regions of the promoter. Since LATs are expressed to high levels in only a small proportion of latently infected neurons it is likely that their expression is tightly regulated during the various stages of latency. In support of this view, transient promoter assays in PC12 cells have identified *cis*-acting sequences which are required for activation by NGF and sodium butyrate, which mediate their affects via the Ras and Raf signalling pathways (Frazier *et al.*, 1996). The in vivo significance of these observations is unclear, although it has been suggested that the upregulation of LATs *via* expression of neurotrophins could function to block reactivation.

LAP1 contains two cAMP response elements (CREs), located at −38 bp (CRE1) and −77 bp (CRE2) relative to the LAT transcription start site, which appear to play an important role in virus reactivation. The CRE1 element has been shown to facilitate both epinephrine-induced reactivation in rabbits and reactivation in mice induced by hyperthermic stress or explantation of ganglia (Bloom *et al.*, 1996, 1997; Marquart *et al.*, 2001). Although these studies suggested that cAMP mediated up-regulation of LATs may be associated with reactivation, it is of interest to note that an *in vitro* neuronal latency model has linked expression of inducible cAMP repressors (ICERs) with downregulation of LATs and subsequent virus reactivation (Colgin *et al.*, 2001). Located adjacent to the LAP1 transcription start site is a binding site for ICP4 which functions to repress expression of LATs during lytic cycle replication. The mechanism by which the LAT promoter remains active during latency and escapes the otherwise global repression which is so efficiently imposed on the latent genome has been subject to much investigation. The observation that LAP1 deleted viruses are able to express 2 kb major LAT during lytic

infection in culture but not during latency in vivo implied the existence of a second promoter between LAP1 and the start of the major LAT intron (Nicosia *et al.*, 1993). A 600 bp fragment within this region was subsequently shown to exhibit promoter activity and was designated LAP2. This second latency associated promoter drives low level reporter gene expression when inserted at an ectopic site in the virus genome. However, despite its designation it would appear that LAP2 functions principally during lytic infection and appears to make a minimal contribution to LATs expression during latency (Chen *et al.*, 1995).

LAP1 is insufficient to mediate long-term latent phase expression, because insertion of reporter genes downstream of LAP1 results in only transient latent phase gene expression. Such studies indicate that although LAP1 contains elements necessary for neuronal expression, additional regulatory sequences are necessary for long-term promoter activity. A long-term expression element (LTE) has been shown to reside downstream of the LAP transcription start site and corresponds to a 1.5 kb fragment which contains an enhancer element and LAP2. Despite extensive efforts it has proven difficult to genetically dissect the downstream LTE sequence. This raises the possibility that the LTE cooperates with LAP1 to direct latent phase transcription (Lachmann and Efstathiou, 1997; Berthomme *et al.*, 2001).

Major LAT ORFs

Major LAT contains two prominent ORFs with the potential to encode proteins of 30 and 15 kd (Fig. 33.1). The lack of conservation of these ORFs between HSV-1 and HSV-2 and the lack of any detectable in vivo latency phenotypes of mutants in which these ORFs are disrupted has suggested that they are unlikely to be of functional significance. Nonetheless, cell lines expressing the 30 kd ORF2 can support the replication of virus mutants defective for IE gene expression and therefore that the ORF can overcome the repression characteristic of quiescent HSV (Thomas *et al.*, 2002). This raises the possibility that this ORF could play a role in reactivation. The significance of these observations remains unclear since there is currently no evidence for the expression of this ORF during infection or following the induction of reactivation from latency; further research in this area is clearly warranted.

Establishment of latency

Specific features of the neuron must be crucial for the interruption of the normal gene expression program during the establishment of latency. The point of arrest in neurons is not known at present, but a number of possibilities, not necessarily mutually exclusive, have been proposed. A description that includes all of the current data and hypotheses in a consistent manner cannot be presented, thus the concepts that are currently favored will be considered separately.

A block to HSV IE transcription

The hypothesis that viral IE transcription fails in neurons has its origins in the observation from tissue culture studies that synthesis of IE proteins is essential for virus replication. HSV mutants with mutations that prevent IE gene expression are not cytotoxic but instead are retained in a quiescent state; therefore, artificial measures to block IE protein production lead to a latency-like interaction in fibroblasts. In mouse ganglia, neurons express either viral antigens or LAT but rarely both during the first few days after infection, suggesting that early events determine the outcome of infection and that latency is incompatible with productive replication (Margolis *et al.*, 1992). Virus mutants that are unable to replicate in neurons still can establish latency. In particular, long term latent promoter activity was observed after inoculation of mice with high doses of HSV-1 mutants that lack the three major transcription activators VP16, ICP0 and ICP4 (Marshall *et al.*, 2000). These mutants enter neurons directly without replicating at a peripheral site. This approach represents the closest available to direct infection of the target cell. The results show that latency can be established in the absence of IE proteins; thus, a natural block to IE transcription in neurons is compatible with the latent state. Ideas on the mechanism by which gene expression in neurons may fail at this stage focus on the requirement for the formation of a TAATGARAT-binding multiprotein complex between VP16, Oct-1 and HCF to initiate IE transcription.

The infecting virion must travel long distances to reach the ganglion, and the structure of the subviral particle that is ultimately delivered to the neuronal nucleus is not known. One idea is that VP16, a tegument protein, fails to be transported to the ganglion with the viral genome, due either to physical loss during retrograde transport or to different uncoating mechanisms in the neuron (Kristie and Roizman, 1988). Alternatively, correct phosphorylation of VP16, especially at serine residue 375 within the Oct-1/HCF recognition domain, is required for its transcriptional activity, and this modification may be affected in neurons (O'Reilly *et al.*, 1997). Absence of functional VP16, even if Oct-1 and HCF are present, would be expected to reduce IE transcription and might, by analogy with

observations in cell cultures, predispose the genome to latency.

Oct-1 is a ubiquitous cellular protein initially defined by its ability to bind to the 'octamer' element ATGCAAAT. The protein participates in a variety of important cellular processes including transcription, and is utilized by many viruses for gene expression or replication. Oct-1 contains a 'POU' domain, which contains the DNA-binding elements and the sites for interaction with many different proteins including VP16. Sensory neurons contain Oct-1 in a form that is functional *in vitro;* thus it is unlikely that absence of this factor underlies a failure of IE transcription (Hagmann *et al.*, 1995). It is possible, however, that neuron-specific members of the POU-containing family interfere with the binding of Oct-1 to viral target sequences. Many POU-containing proteins bind to TAATGARAT elements in IE promoters but do not interact with VP16, and it would be expected that such proteins could compete with Oct-1 and thereby block gene activation (Latchman, 1999). Rodent Oct-1 varies from the human protein specifically at a few residues that are important for binding of human Oct-1 to VP16 and HCF (Cleary *et al.*, 1993). Thus, VP16 forms the multiprotein complex less efficiently with murine Oct-1, raising concerns about the relevance of the mouse models of latency. Possibly, latency is relatively favored over lytic replication in mice compared with humans. Murine Oct-1 must function to some extent in vivo, however, because HSV-1 VP16 mutants are severely attenuated for replication in mice; if Oct-1 were inactive, the absence of VP16 function would probably be inconsequential.

HCF is a large cellular protein of 2035 amino acids that undergoes internal proteolytic cleavage but nonetheless can participate in activation of transcription with only a heterodimer of the critical N- and C- terminal fragments. The N-terminal portion contains six repeats with homology to the *Drosophila* protein Kelch, that are predicted to form a propeller-like structure which binds VP16 (Wilson *et al.*, 1993). One major function of HCF appears to be stabilization of the Oct-1/VP16/HCF complex, but more recent studies suggest that HCF itself contains activating regions that may contribute to stimulation of gene expression (Lociano and Wilson, 2002). In proliferating cells HCF is associated with chromatin and is important for cell division, since a cell line harboring a temperature sensitive mutation in HCF arrests predominantly at G0/G1 upon shift to the non-permissive temperature. In sensory neurons in vivo, HCF appears to be cytoplasmic, possibly reflecting the non-dividing state of the cells (Kristie *et al.*, 1999). This localization, if maintained after infection, would prevent activation of IE transcription through the VP16-mediated pathway. Cellular proteins have been identified that, like VP16, contain the short motif $^{D}/_{E}$ HXY which interacts with the Kelch domain of HCF. One of these, named LZIP or Luman, is cytoplasmic in tissue culture cells and, when over-expressed, redistributes HCF from the nucleus to the endoplasmic reticulum (ER) (Freiman and Herr, 1997; Lu and Misra, 2000). Transfected tissue culture cells expressing Luman are impaired for productive HSV-1 replication, presumably because HCF is sequestered at the ER. Another HCF-binding protein, Zhangfei, is selectively expressed in human neurons and also blocks HSV-1 replication when ectopically expressed in tissue culture cells, possibly by counteracting VP16 (Akhova *et al.*, 2005). Clearly, if Luman, Zhangfei or other HCF-binding proteins are present in neurons, activation of IE transcription may be impaired, due to relocation of HCF to the ER, to competition for VP16-binding sites, or to interaction with VP16.

Role of LAT in the establishment of latency

Expression of LAT is not essential for any phase of latency, but there is considerable evidence that it plays a modulatory role. When LAT+ and LAT− viruses are compared virus production at the periphery and in the ganglion is generally equivalent, although early studies suggested that LAT− mutants produce greater quantities of lytic transcripts and proteins in neurons (Garber *et al.*, 1997). In general, however, LAT− mutants reactivate inefficiently and much experimentation has centered on whether this reflects a defect in reactivation *per se* or is a consequence of reduced ability to establish latency. Analysis of ganglionic viral DNA contents by direct PCR yields equivocal results, with some investigators reporting a deficit of around threefold and others detecting no significant difference. Errors in these estimations are inherently large, thus relevant differences might not score as statistically significant. The application of CXA revealed that corneal infection with LAT− mutants results in approximately threefold fewer latently infected neurons in trigeminal ganglia, although the HSV-1 genome content distribution within cells was indistinguishable from that of mice infected with a LAT+ virus (Thompson and Sawtell, 1997). These results suggest that LAT affects the number of neurons that ultimately harbor the latent genome rather than copy number within individual cells, and this conclusion is supported by investigation of the effect of LAT on neuronal survival (Perng *et al.*, 2000). Infected rabbit ganglia exhibited greater neuronal apoptosis after infection with a LAT− virus than with a LAT+ counterpart. This effect was maximal at 7 days post-infection, and, surprisingly, few apoptotic neurons were detected at 3 days post-infection, when virus

replication was at its peak. It is proposed that LAT has an anti-apoptotic activity that could result in a greater number of neurons surviving in animals infected with the LAT+ virus, thereby increasing establishment of latency. In mice, the basic observation that LAT improves neuronal survival also holds, although there is currently debate concerning whether death is through apoptosis or an alternative route (Thompson and Sawtell, 2001; Ahmed *et al.*, 2002). In tissue culture cells, expression of LAT from transfected plasmids or viruses inhibits apoptosis induced by toxic agents or by virus infection itself, supporting the idea of an anti-apoptotic role (Inman *et al.*, 2001). Furthermore, recent data showing that a miRNA encoded by the HSV-1 LAT gene regulates apoptosis induction by modulating TGF-beta signalling adds considerable support to the view that an important biological function of LATs is to prevent neuronal apoptosis during latency establishment and/or reactivation (Gupta *et al.*, 2006).

LAT has been proposed to block IE gene expression, possibly by antisense inhibition of ICP0 synthesis, an hypothesis that could explain the greater toxicity of LAT-mutants for neurons. Cultured neuroblastoma cells transformed stably to express the 2 kb LAT exhibited reduced permissiveness to HSV-1 infection and a reduction in the levels of all IE-specific mRNAs, suggesting an inhibitory effect of LAT on IE RNA production through a *trans*-acting mechanism (Mador *et al.*, 1998). However, no reduction in ICP0-specific transcript or protein levels was found in human 293T cells engineered to express 2 kb LAT (Burton *et al.*, 2003).

Alternative models for establishment of latency

In studies with cultured neurons ICP0 was not detected in the nucleus, even though ICP0-specific RNA was expressed (Chen *et al.*, 2000). The reasons for the failure to detect the protein are not clear, although post-transcriptional mechanisms are implicated. The absence of ICP0 might predispose the virus to latency.

All models for the establishment of latency are complicated by the fact that neurons are not inherently resistant to HSV infection because a proportion is able to support productive replication during the first few days after inoculation of animals. There is some evidence that specific neuronal subtypes may differ in susceptibility, but an absolute distinction between permissive and non-permissive cells has not been made to date. Most of the viral DNA produced during the acute phase is eliminated by a rapidly evolving immune response; however, there remains the possibility that some of the latent genomes are derived from replicated molecules rather than transport from peripheral sites. Studies with TK-mutants argue against this hypothesis for snout and corneal inoculation of mice, but in a flank inoculation model evidence was obtained for retention of replicated DNA in neurons that directly innervate the site of infection (Simmons *et al.*, 1992).

An all-encompassing model does not exist to describe the molecular basis for the establishment of latency. If the idea of an early decision between lytic infection and latency, with a primary block at the level of IE gene expression, is accepted, then there would be no apoptotic stimulus (in the form of *de novo* synthesized viral proteins) to the neuron. This is difficult to reconcile with the hypothesis that LAT antagonizes a response to the presence of viral proteins, which presupposes that the gene expression program proceeds past the IE stage. Possibly, there is heterogeneity in the responses of individual infected neurons, such that some escape an IE block but are arrested at a later stage by LAT. Understanding the cause of neuronal death in infected ganglia is critical to a resolution of these issues. The effect of LAT on the establishment of latency is anatomical site-specific, since LAT− mutants apparently show no difference from LAT+ HSV-1 when latency in dorsal root ganglia is examined after inoculation of the footpad (Sawtell and Thompson, 1992a).

Maintenance of latency

The stability of the latent state, together with the failure to detect viral gene expression apart from that of LAT, supports the concept that the majority of the genome is in a silent state that can be reversed only by specific triggers. Studies in the mouse using sensitive RT-PCR, however, demonstrated that transcripts from the ICP4 and TK regions of the genome could be detected in ganglia during latency (Kramer *et al.*, 1998). This observation is supported by experiments in which sections from many mouse ganglia were analyzed by ISH (Feldman *et al.*, 2002). Approximately one neuron per 10 sections was found to be positive for transcripts representing the lytic genes ICP4, TK and glycoprotein C. In addition, antigen positive neurons were detected at approximately the same frequency, and these cells were surrounded by an immune infiltrate. The most reasonable explanation for the results is that a few neurons support viral gene expression in the mouse, a view that is supported by the finding that interferon gamma and CD8+ T cells are present in murine ganglia at latent times, suggesting that active immune surveillance may operate to maintain latency (Cantin *et al.*, 1995; Khanna *et al.*, 2003). Further discussion of these results is given elsewhere in this volume. Therefore, although the majority of

latent genomes are retained in an untranscribed state, the possibility exists that some neurons express HSV-specific proteins and are prevented from producing virus by host immune responses.

Reactivation

Since viral gene products characteristic of the lytic cycle cannot, in general, be detected in latently infected neurons, cellular mechanisms must be important for reactivation. The crucial cellular events are not understood at the molecular level and are still vaguely described as applying 'stress' to the neuron. Furthermore, the models for reactivation may rely on very different cellular stimuli and hence the mechanisms involved may vary between both animals and systems. For instance, explantation is probably a more severe stress than in vivo treatments. A further serious complication arises from the fact that reactivation is an inefficient process with only a small proportion of the neurons that harbor viral genomes responding by production of virus. This means that the genomes detected at a gross level during latency may not represent those able to reactivate, with the latter possibly forming a small subset of the total. In addition, in a comparison between HSV-1 strains that differ in their abilities to respond to hyperthermia in vivo, the efficiency of reactivation correlated with the genome copy number distribution but not the number of neurons harboring latent virus (Sawtell, 1998). Therefore, the neurons containing large amounts of HSV DNA may be more susceptible to reactivation stimuli in vivo.

Models for reactivation depend critically on understanding the mechanism of establishment of latency. Thus, if the view is taken that a block in IE transcription leads to establishment, the route to reactivation can be subdivided into two basic concepts, depending on the consequences of the IE block. If, as in fibroblast models, failure of IE gene expression results in conversion of the genome into a quiescent state that is disrupted by ICP0 but is unresponsive to changes in cell physiology such as activation of signal transduction pathways, it follows that reactivation must be provoked either by the action of cellular proteins that mimic the activity of ICP0 or by induction of ICP0 synthesis. An alternative, more popular, view is that viral promoters are not repressed thoroughly, as in fibroblasts, but are inactive and potentially responsive to cellular signals provided by reactivation stimuli. The two models overlap if the genome is generally repressed but the ICP0 promoter specifically escapes repression. In this case, reactivation stimuli would initially be targeted to the ICP0 promoter, with the subsequent reversal of genome repression by the ICP0 protein. A role for LAT in reactivation is suggested by a number of experimental observations, although the interpretation of the data again depends on the events leading to establishment.

The role of ICP0 in reactivation

ICP0 was first characterized as a transcription activator that is not sequence-specific, but recent studies have shown that its primary mode of action is as an ubiquitin E3 ligase that mediates the targeted proteolysis of cellular proteins, particularly those of the nuclear structures known as ND10 (Everett, 2000; Van Sant *et al.*, 2001; Boutell *et al.*, 2002). Indeed, ICP0 rapidly and effectively mediates the disruption of all ND10 in the cell, with accompanying degradation of many of the component proteins. Since transcriptionally active input HSV genomes initially associate with ND10, it is thought that ICP0 creates an environment that is conducive to transcription, probably by directing the destruction of cellular repressors. Histone deacetylases (HDACs) promote the formation of inactive chromatin, thus it is interesting that ICP0 interacts with HDACs 4, 5 and 7 (Lomonte *et al.*, 2004). ICP0 also dissociates HDAC 1 and 2 from CoREST/REST, a protein complex that represses transcription, thereby possibly relieving repression (Gu *et al.*, 2005). These data suggest an important role for ICP0 in antagonizing histone-mediated gene silencing.The dramatic reversal of the quiescent state by ICP0 in cell culture suggests that this protein may be important for reactivation of latent HSV. Early in vivo studies showed that ICP0-deficient mutants were impaired for latency, as measured by reactivation efficiency after explantation, but it was not possible to distinguish between a true effect on reactivation and inefficient establishment due to the known reduction in replication at the periphery and in the ganglion. Immunosuppression of mice enables ICP0 null mutants to establish latency as efficiently as wild-type virus as judged by latent genome copy number, and ICP0 null mutants exhibit reduced reactivation efficiency in the explant model even when viral DNA loads in the ganglia are equivalent (Halford and Schaffer, 2001). ICP0 is therefore important for explant reactivation, but the exact stage at which it functions is unclear. Explantation might specifically induce the synthesis of ICP0, but an alternative interpretation is that ICP0 merely improves the replication, and hence detection, of HSV-1 once the reactivation stimulus has acted. The former hypothesis predicts that the promoter, or other important sequences controlling ICP0, contains elements that respond to reactivation stimuli.

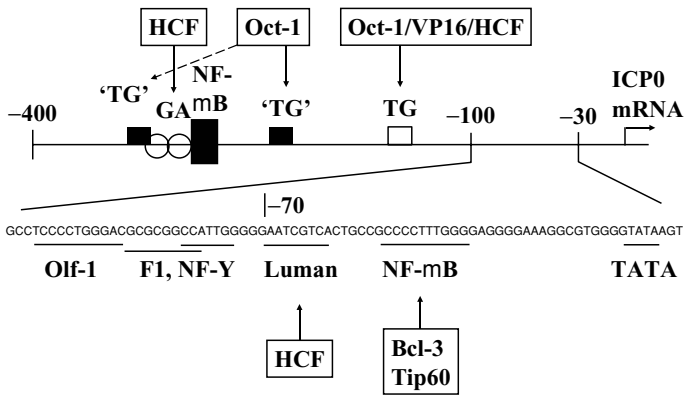

Fig. 33.2. Binding sites for transcription factors in the ICP0 promoter. The region to nucleotide −400 is shown, with the −100 to −30 sequences expanded. The major TAATGARAT element (TG), and two TAATGARAT homologies that have not been demonstrated to bind VP16 and HCF ('TG') are shown. Other sites in the −400 to −100 region predicted to bind GABP (GA; two sites) and NF-κB are also shown. There are functional data to support the binding of Olf-1, F1 plus NF-Y, and Luman to the indicated sites. The CCCCTTTGGGG motif at −57 is predicted to bind NF-κB on the basis of sequence homology.

The ICP0 promoter as a possible target for reactivation signals

The ICP0 promoter has many motifs, in addition to the TAATGARATs, that bind transcription factors, and these sites might be targets for reactivation stimuli (Fig. 33.2). Nucleotides –79 to –97 bind Olf-1, a neuron-specific factor that activates transcription (Devireddy and Jones, 2000), and sequences between –74 and –89 are recognized by two proteins, NF-Y and one of unknown identity (named F1), in the human neuroblastoma line IMR-32 (O'Rourke and O'Hare, 1993). Gene array analysis demonstrated that explantation of ganglia induces the synthesis of Bcl-3 (as well as other gene products) in neurons (Tsavachidou *et al.*, 2001). This is interesting because Bcl-3 associates with a dimer of the p50 subunit of NF-κB, and the ICP0 promoter contains NF-κB consensus binding sites at –51 and –273. Phosphorylation influences the binding of Bcl-3 to p50, thus explantation may activate kinases that promote the interaction of these proteins. Bcl-3 also interacts with Tip60, a histone acetylase, and therefore may mediate its effects by recruiting this protein and modifying chromatin structure at the ICP0 promoter. In ganglia, HCF is found in the cytoplasm of neurons but is transported to the nucleus within 20 minutes of explantation (Kristie *et al.*, 1999). If the HSV genome, or strategic regions such as the ICP0 promoter, is available for transcription then HCF, by virtue of its intrinsic activation domain, could trigger the viral gene expression program. This hypothesis requires that a mechanism exists for localizing HCF to the promoter, and binding to Oct-1 is the obvious candidate. However, HCF interacts with the ETS family member GABP, which binds to motifs of consensus CGGAAR (Vogel and Kristie, 2000). There are GABP recognition sites in the ICP0 promoter, and these might direct activation of transcription through a GABP/HCF complex. Another HCF-binding protein, Luman, is cytoplasmic in tissue culture cells but is, in essence, a basic leucine zipper transcription factor of the ATF/CREB family that can bind to CREs and activate transcription in an HCF-dependent manner (Lu and Misra, 2000). In tissue culture cells, Luman is released from the ER by the action of the site 1 protease, an enzyme that catalyzes the regulated intramembrane proteolysis (RIP) of membrane-bound transcription factors, releasing them for transport to the nucleus and activation of transcription (Raggo *et al.*, 2002). Various stress stimuli trigger RIP, thus reactivation signals may result in the release of Luman from the ER of neurons, thereby relocating a complex of this protein plus HCF to the nucleus. The proposed site of action is a CRE at position –67 in the ICP0 promoter.

Hypotheses on the significance of transcription factor binding to the ICP0 promoter must include the possibility that such binding may be relevant to replication in neurons at the early stages of infection rather than to reactivation. In addition, all of the above ideas must take account of studies on an HSV-1 mutant deleted for nucleotides –70 to –420 in the ICP0 promoter, thus lacking most of the factor-binding sequences mentioned above (Davido and Leib, 1996). This mutant established latency and displayed normal reactivation efficiency in the explantation system even though replication in cell culture was impaired, suggesting that the region between –70 and –420 in the ICP0 promoter, which includes most of the important known elements controlling expression in cell culture systems, does not contain critical target sequences for explant reactivation of latent virus. Notably, the NF-κB binding site at –51 and the CRE at –67 lie outwith the dispensable region.

Cellular reactivation signals

Among the many changes that occur in neurons following explantation of ganglia, the cyclin-dependent kinases cdk2, cdk4 and cdk7 exhibit alterations in abundance and location (Davido *et al.*, 2002). Increases in the level of cdk2 were observed, and this enzyme was found predominantly in the nucleus. In the case of cdk4, the protein was found mainly in the cytoplasm immediately after plating of explanted ganglia, but became nuclear during culture. A dramatic drop in cdk7 levels occurred within the first day of explant. HSV-1-specific antigens were found exclusively in those neurons

containing nuclear cdk2 and cdk4, suggesting that changes in the kinases might be required for reactivation. Roscovitine, an inhibitor of cdk2 that blocks HSV-1 replication in tissue culture, also prevented virus reactivation. Furthermore, no HSV-1 antigen reactivity could be detected in the presence of roscovitine, suggesting that the inhibitor blocks reactivation at an early stage rather than during spread of virus in the explanted ganglion. In tissue culture systems, roscovitine affects many aspects of HSV-1 replication, but it is noteworthy that the compound blocks the function of ICP0 due to alteration of post-translational modification. Therefore, cdk2 and cdk4 may be important for reactivation due to their roles in ensuring the activity of ICP0. In cultured primary rat neurons, withdrawal of NGF results in the rapid resumption of virus replication, to an extent mimicking one of the effects of explantation, in which the in vivo supply of NGF is disrupted (Wilcox and Johnson, 1988). Inhibition of deacetylases also reactivates latent virus in cultured neurons, suggesting a requirement for modification of chromatin structure (Arthur *et al.*, 2001). Treatment of cells with agents that activate signal transduction pathways through cAMP-mediated mechanisms is effective, and recent studies have indicated an involvement of inducible cAMP early repressors (ICER) in this process (Colgin *et al.*, 2001). ICER can heterodimerize with CREB/ATF transcription family members that mediate the transcriptional changes induced by cAMP, but since ICER lacks an activation domain the complexes act as repressors when bound to CREs. Expression of ICER itself is activated by cAMP and, intriguingly, by heat stress of neurons. Crucially, reactivation of latent HSV-1 was induced by infection of neuronal cultures with an adenovirus recombinant expressing ICER. In concert with reactivation, the levels of LAT decreased, leading to the suggestion that the known CREs in the LAT promoter mediate repression by ICER. This observation is difficult to reconcile with suggestions of a positive role for LAT in reactivation, as discussed below. However, in a model that uses cultured cells from dissociated ganglia of latently infected mice, transient heat shock or addition of dexamethasone induced reactivation but elevation of cAMP levels did not (Halford *et al.*, 1996). The nature of the reactivating stimuli therefore differs in the various cell culture systems currently available.

The role of LAT in reactivation

Early work ascertained that, in most cases, LAT− virus mutants reactivate less efficiently than their LAT+ counterparts. This observation was made in mice, for both explant and in vivo reactivation, in rabbits, and in guinea pigs infected with HSV-2. The conclusion that LAT has a role in reactivation is therefore widely accepted. This assumption is complicated by the findings, discussed above, that LAT− mutants establish latency less efficiently in some systems; clearly if fewer neurons harbor HSV genomes, a lower reactivation potential would be expected. Studies in mice, analyzing neuronal DNA contents by CXA, concluded that the impaired reactivation of LAT− mutants in trigeminal ganglia can be entirely accounted for by reduced establishment of latency (Thompson and Sawtell, 1997). Tellingly, it was possible to increase the efficiency of establishment by LAT− mutants to that of wild-type HSV-1 if hyperthermic treatment was applied during the first three days after infection. An equivalent rise in in vivo reactivation frequency to wild-type levels accompanied the increased establishment, strongly suggesting that the primary role of LAT in the mouse trigeminal ganglion is at the level of establishment of latency. In the rabbit eye model, LAT− mutants exhibit reduced efficiency of both spontaneous and induced reactivation. Analysis of latent DNA levels is difficult in this model, however, and although most studies conclude that LAT− and LAT+ mutants establish latency with equivalent efficiencies, variation in the data could obscure a three-fold difference. The question of whether LAT affects establishment or reactivation, or both, in the rabbit remains open. Intriguingly, replacement of the LAT region of HSV-2 with the equivalent region from HSV-1 revealed a role of LAT in anatomical-site specificity of reactivation (Yoshikawa *et al.*, 1996). The recombinant acquired HSV-1-like characteristics, displaying an increased response to iontophoresis of epinephrine in the rabbit but a reduced reactivation frequency in the guinea pig. Only the first 1.5 kb of the mLAT transcript, representing the LAP2 region and part of the stable LAT, is required for efficient spontaneous reactivation in the rabbit (Perng *et al.*, 1996). Re-introduction of sequences encoding this region, plus 1.8 kbp of the LAT promoter, between the UL37 and UL38 coding sequences of a LAT− mutant restored the defect and resulted in a virus exhibiting normal reactivation phenotype. Comparison between strains suggested that none of the ORFs that can be detected in the 1.5 kbp fragment is functionally important for reactivation (Drolet *et al.*, 1998). In addition, inhibition of apoptosis by HSV-1 LAT in tissue culture cells also maps to the 1.5 kbp region (Inman *et al.*, 2001). Thus, in the rabbit, the 1.5 kbp region is thought to mediate increased spontaneous reactivation by virtue of its anti-apoptotic activity. This view is strengthened by the finding that the reduced reactivation efficiency of LAT-viruses can be reversed by insertion of sequences that encode a baculovirus anti-apoptotic protein (Jin *et al.*, 2005). Therefore, the anti-apoptotic function of the LAT region may be important for prolonging survival of the reactivating cells and increasing the production of infectious virus. Deletion of a subfragment of the 1.5 kbp region, consisting of a part of LAP2, dramatically

reduced the efficiency of epinephrine-induced reactivation in the rabbit (Bloom *et al.*, 1996). This deleted region lies within the mLAT region and does not affect the accumulation of major LAT; thus, presumably either the expression of a transcript or a *cis* effect accounts for the activity of the LAP2-derived element and probably the entire 1.5 kbp region.

All of the ideas described above on the mechanism of reactivation assume that latency is essentially "static," with a switch required to reverse the silencing of the genome. The alternative "dynamic" model, in which continual low level production of virus occurs with lesions only occurring sporadically, does not readily fit with known ideas of viral gene expression but may be consistent with the detection of CD8+ T cells in ganglia (Khanna *et al.*, 2003). The use of PCR has demonstrated that aymptomatic shedding of HSV-1 and HSV-2 occurs with surprisingly high frequency in humans (Wald *et al.*, 1997), suggesting that the dynamic model, an interaction which could be envisaged as a slow persistent infection, deserves consideration.

Concluding remarks

Latency is clearly very complex at the molecular level, and the difficulties inherent in the model systems ensure that it will not be unraveled easily. The non-uniformity of latency, in terms of viral genome copy number, LAT expression and nature of reactivation stimulus, may be of fundamental benefit to the virus. If latency was uniform, a single stimulus might induce reactivation in the entire latent reservoir and result in clearance of the virus from the host. Perhaps the different virus/cell interactions respond to different host signals, explaining why it has been so difficult to arrive at a simple model for the molecular basis of latency.

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Immunobiology and host response

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Introduction

Herpesviruses began to evolve prior to the development of acquired immunity (Arzul *et al.*, 2002). It is therefore likely that evasion of innate immunity is an ancient function of alphaherpesviruses. Additional immune evasion functions have developed to adapt to the diverse repertoires of B- and T-cell immune receptors that characterize acquired immunity (Roizman and Pellet, 2001; Littman *et al.*, 1999). Immune evasion is covered in detail elsewhere in this volume. The innate and acquired immune responses to HSV are relevant to preventative and therapeutic vaccines for HSV, HSV-induced immunopathology, and the use of modified HSV for gene or cancer therapy. While human studies are, of necessity, observational or ex vivo in nature and seldom access sites of neuronal latency, we review them in detail because of their medical relevance. The excellent tools available for murine studies, including exquisite control of the DNA sequence of HSV challenge strains, and of the phenotype and genotype of recipient animals, are yielding dramatic new insights as well. Reactivation of HSV from neuronal latency is less frequent in mice than in humans, limiting immunologic studies of this challenging phenomenon. Readers are referred to excellent reviews (Schmid and Rouse, 1992; Nash, 2000; Lopez *et al.*, 1993, Simmons *et al.*, 1992; Kohl,1992) for models and materials that cannot be covered in detail.

HSV interactions with dendritic cells

Dendritic cells (DC) are a major link between innate and acquired immunity. DC are mobile cells that can potently initiate acquired immunity. Priming of the HSV-specific CD8 response occurs promptly and vigorously, suggesting the involvement of DC. After HSV-1 footpad inoculation, draining lymph nodes (DLN) of C57BL/6 mice are infiltrated by large, activated (CD44+), CD8+, CD62L− cells that express Vβ10. In these mice, the CD8 T-cell response to HSV-1 is dominated by Vβ10+, K^b-restricted, HSV-1 $gB_{498–505}$-specific cells (Cose *et al.*, 1995; Wallace *et al.*, 1999). HSV-specific cells are detectable in DLN by tetramer staining by day 2 (Coles *et al.*, 2002), and peak at day 5 at about 5% of the total DLN CD8+ cells (Cose *et al.*, 1997; Jones *et al.*, 2000; Coles *et al.*, 2002). The low direct ex vivo HSV-specific cytotoxic T-lymphocyte CTL activity of DLN cells increases about 40-fold during simple "holding" of the cells in vitro for a few days. This functional maturation correlates with a 35-fold increase in the number of HSV-specific CD8+ cells during culture. These results imply profound in vivo stimulation of naive HSV-specific cells in vivo, which continues in vitro.

Infectious virus was not detected in the DLN (Jones *et al.*, 2000), suggesting that mobile antigen presenting cells (APC) acquire HSV antigen in the periphery and cross-present antigen to naïve CD8 T-cells in DLN. While cross-priming of HSV to naïve CD8 T-cells has not been reconstituted in vitro, fibroblastoid cells, as expected, were not competent for this function (Mueller *et al.*, 2003). The quickness of CD8 T-cell priming after HSV infection is impressive. Naïve wild-type mice were adoptively treated with syngeneic, naïve HSV-specific CD8 cells (from transgenic mice) and then infected. The HSV antigen is presented in the DLN by 6 hours after footpad infection, as detected by CD69 expression or a reporter gene, with the first cell division occurring within 24 hours. The HSV-specific cells gained effector CTL function simultaneously with replication. De novo HSV protein synthesis, rather than delivery of protein in the inoculum, was required for naïve T-cell stimulation, as demonstrated

with mutant viruses incapable of encoding the dominant epitope. Detection of viral DNA in the DLN again did not correlate with the priming of HSV-TCR (T-cell receptor) cells (Coles *et al.*, 2002).

DC subsets can be defined by tissue distribution, morphology, surface markers (which can differ between species), and responses to pathogens or other stimuli. To study which were DC involved in priming for HSV, DLN cells from two days after footpad infection were fractionated and admixed with naïve HSV-TCR cells (Smith *et al.*, 2003). B-cells, T-cells, CD11b+ macrophages, and Langerhans cells were not active APC. Depletion of cells expressing CD11c, CD8α, or DEC205 abrogated antigen presentation. The biologically active cells were CD8α+ CD45− cells, distinct from CD45+ plasmacytoid DC (O'Keeffe *et al.*, 2002). These "conventional" DC (CD8α+ in mice) are efficient cross-presenters (Iyoda *et al.*, 2002; den Haan *et al.*, 2000). While the DLN cells contained HSV DNA (Jones *et al.*, 2000; Smith *et al.*, 2003), other studies in this system (Jones *et al.*, 2000) showed no infectious virus. Similar studies were performed after flank skin inoculation with HSV, which results in a strictly epidermal infection. DC again accumulated in DLN that can prime naïve HSV-specific cells. No HSV DNA signal was detected in the active DLN cells (Mueller *et al.*, 2002). Fractionation studies (Allan *et al.*, 2003) revealed that the active APC were "conventional" CD11c+, CD8αhigh, CD205+, CD45RA− DC. Langerhans cells and plasmacytoid DC were detected in DLN, but did not have direct APC activity. Recently, the Carbone and Heath group determined that migratory DC do not directly present HSV antigen to naïve T-cells. Rather, DLN-resident CD8α+ DC appear to acquire antigen from migratory DC (Allan *et al.*, 2006). Local plasmacytoid DC-like cells and interferon-alpha may also play an important role in assisting priming of HSV-specific CD8 CTL responses (Yoneyama *et al.*, 2005). Taken together, multiple DC subsets appear to work together to prime cellular immunity after cutaneous HSV infection (Randolph, 2006).

The DC implicated in the priming of murine CD4 responses after HSV-2 vaginal inoculation may have a slightly different phenotype. Infection of steroid-treated mice with a *tk*- HSV-2 strain is confined to the epithelium. Zhao *et al.* found that CD11b+, CD11c+ cells migrated to the submucosa in areas subjacent to HSV-2 infection. DLN CD11c+ cells displayed up-regulation of costimulatory molecules for several days after inoculation. By day 2, DLN CD11c+ cells specifically stimulated HSV-specific CD4 T-cells, presumably due to presentation of HSV-2 antigen acquired in vivo. Fractionation showed that CD11c+, CD11b+ cells, but not B cells, were active APC. In contrast to the CD8 T-cell priming studies, the APC were CD8α−. Again, the active cells did not have features of Langerhans or plasmacytoid DC. No HSV DNA was detected in the DLN cells (Zhao *et al.*, 2003). Combined, these recent studies of priming after HSV infection demonstrate that specific DC subsets prime HSV-specific CD4 and CD8 T-cells in the apparent absence of their direct infection by HSV.

The APC involved in T-cell priming during primary HSV infections of humans are unknown. Langerhans cell numbers are decreased in HSV-infected skin, possibly consistent with their emigration and a role in antigen presentation (Memar *et al.*, 1995). Skin-derived Langerhans cells have APC function for HSV-specific memory HLA class II-restricted (CD4) responses in vitro (Vestey *et al.*, 1990). The ability of other cell types to present HSV antigen in the recall context is reviewed below.

In some viral infections, specific interactions with dendritic cells, such as direct infection, have roles in pathogenesis (Servet-Delprat *et al.*, 2003). Several groups have examined HSV infection of DC and the activity of immune evasion functions in these cells (Becker, 2003.) Human in vitro-generated myeloid DC express HSV entry receptors (Salio *et al.*, 1999) and can be productively infected by a clinical HSV-1 strain (Mikloska *et al.*, 2001). Efficient entry, but restricted immediate early gene expression, and little or no production of daughter virus was observed with various lab strains of HSV-1 (Rong *et al.*, 2003; Kruse *et al.*, 2000; Pollara *et al.*, 2003; Samady *et al.*, 2003). HSV is pathogenic in both murine and human DC even in the absence of productive infection (Jones *et al.*, 2003; Samady *et al.*, 2003; Pollara *et al.*, 2003). There is more agreement that HSV infection generally inhibits DC maturation and function. Addition of infectious HSV generally blocks LPS-mediated maturation of myeloid DC, as measured by up-regulation of T-cell co-stimulatory molecules, with some evidence that "bystander" uninfected DC may be activated to mature by factors released from infected DC (Salio *et al.*, 1999; Samady *et al.*, 2003; Pollara *et al.*, 2003). Bystander DC were not adversely affected in terms of cytokine secretion and T-cell stimulatory capacity for third-party responses, while infected cells were markedly functionally impaired (Salio *et al.*, 1999; Pollara *et al.*, 2003). Infection of mature myeloid DC can specifically down-regulate CD83 (Kruse *et al.*, 2000). Some genes responsible for this inhibition have been identified. In HSV-1, *vhs* (unique long gene 41, *UL41*) may be involved (Samady *et al.*, 2003). For HSV-1, deletion of vhs and US12 (encoding unique short gene 12, infected cell protein 47, ICP47) has been reported to improve DC antigen presentation (Sun *et al.*, 2003). The complex, mainly inhibitory effects of HSV on DC function were examined in the context of stimulating recall T-cell responses to HSV itself. Lower MOIs permitted detection of memory

responses, which were abrogated by high-dose infection of human myeloid DC (Pollara *et al.*, 2003).

CD8 T-cell responses to HSV

The long-term consequences of primary infection include immunologic priming and latent infection of ganglia. Events of immunologic interest in the ganglia are reviewed first, followed by a discussion of memory CD8 responses in the blood and peripheral tissues. While mice do not have spontaneous HSV recurrences, virologic and immunologic data are consistent with chronic, low level HSV gene expression and immune recognition in dorsal root ganglia (DRG). High subclinical HSV shedding rates in humans are consistent with chronic or very frequent, intermittent reactivation (4). HSV may be fundamentally less tightly controlled in human than in murine ganglia.

After recovery from HSV-1 infection, latently infected murine ganglia show persistent evidence of inflammation. Feldman *et al.* 2002) studied trigeminal ganglia (TG) of mice 5 to 7 weeks after recovery from ocular inoculation with HSV-1. mRNA for lytic HSV genes were localized to rare neuron-like cells surrounded by mononuclear leukocytes, while LAT (+) neurons lacked this infiltrate. The ratio of LAT RNA (+) to lytic RNA (+) cells was about 5000. Late protein (gC) was detected by immunohistochemistry in rare neuron-like cells. These data extend RT-PCR analyses of latently infected ganglia that detect lytic HSV-1 mRNA (Kramer and Coen, 1995). Latently infected ganglia are also enriched for mRNA encoding pro-inflammatory and lymphocyte-specific proteins such as IFN-γ. The cellular source of this IFN-γ mRNA is somewhat obscure (Tscharke and Simmons, 1999) despite the detection of HSV-specific, IFN-γ-producing cells in infected ganglia (below). Levels of these host response transcripts do not strictly correlate with the ability to reactivate: mutant *tk*- strains, which cannot reactivate to make infectious virus, still lead to persistent inflammation (Chen *et al.*, 2000). Possibly, specific viral proteins, or HSV DNA, which is rich in potentially immunostimulatory CpG sequences (McGeoch *et al.*, 1988), stimulate inflammation in the absence of complete reactivation. Therapy that interrupts HSV DNA replication reduces (but does not eliminate) inflammation in murine TG latently infected with a different HSV-1 strain (Halford *et al.*, 1997).

The CD8 response in murine ganglia can be separated into acute and latent phases. In A/J mice, the earliest cells infiltrating the TG after corneal HSV-1 strain RE infection are NK-like cells and macrophages (Liu *et al.*, 1996). Virus is cleared by day 7; TCR γδ cells start to appear at this time. The CD8 infiltrate peaks on day 12, most after viral antigen becomes undetectable. In this model, a significant number of CD4, CD8, TCR γδ, macrophage-like, and NK-like cells, and cells positive for TNF, persist in the ganglia for up to 90 days. After flank scarification of mice, CD8+ cells are involved in control of HSV-1 replication in the draining ganglia (Simmons and Tscharke, 1992). Temporally, there is a good correlation between CD8 cell infiltration and viral control (Speck and Simmons, 1998). MHC class I molecules are observed to be up-regulated on ganglionic cells during this phase, and neuronal cell death is not observed (Pereira and Simmons, 1999; Speck and Simmons, 1998; Pereira *et al.*, 1994).

CD8+ cells may "monitor" the HSV-1-infected murine ganglia and contribute to the maintenance of clinical latency. Explanted, HSV-1 latently-infected TG contain endogenous CD8+ cells that suppress reactivation. Exogenous, immune CD8+ T-cells can serve the same function in an MHC-restricted fashion (Liu *et al.*, 2000; Khanna *et al.*, 2003). A non-lytic mechanism is suggested by the continuing presence of HSV genomes (presumably in latently infected neurons) in "suppressed" cultures. IFN-γ may exert an antiviral effect. The release of IFN-γ from TG in culture is inhibited by acyclovir, implying that lymphocyte recognition of viral protein is a step in lymphokine secretion. In addition, exogenous IFN-γ protects against reactivation from latency in ganglionic explants in a model which includes initial acyclovir blockade (Liu *et al.*, 2001). Direct effects of IFN-γ on neurons, and indirect effects mediated by CD8+ cells, were both detected. Another possible effector molecule is granzyme A, a constituent of CD8 T-cell and NK cell granules (Lieberman and Fan, 2003). Animals deficient in granzyme A show decreased viral clearance from draining DRG after peripheral HSV-1 inoculation (Pereira *et al.*, 2000).

Direct evidence for involvement of HSV-1-specific "classic" CD8 T-cells in viral control in TG has been obtained in C57BL/6 mice. Cells recognizing the dominant K^b-restricted, HSV-1 $gB_{498–505}$ epitope infiltrate TG 2 to 5 weeks after ocular HSV-1 infection, as shown by tetramer staining (in situ and after dissociation of TG) and IFN-γ responses (Khanna *et al.*, 2003). Interestingly, these cells appear to become increasing activated from day 14 to day 34. The functional antiviral activity of the ganglionic CD8 cells appeared to wane somewhat by day 34 (Liu *et al.*, 2000). The duration of ganglionic localization is of interest, given the chronicity of HSV infections in humans. It is not known whether the HSV-specific CD8 cells are reacting to low levels of lytic protein expression, and/or somehow sense, or assist with the maintenance of, latent gene transcription. The factors involved in T-cell trafficking to

ganglia are unknown. Taken together, these data are consistent with a model in which HSV-specific CD8+ T-cells are persistently localized to HSV-infected ganglia. Recently, the group of Verjans and Osterhaus (Osterhaus *et al.*, 2006) has demonstrated that human trigeminal ganglia latently infected by HSV-1, contain HSV-specific CD8 T-cells capable of producing IFN-γ. It will be of great interest to determine the effector functions, phenotype, and fine specificity of these T-cells. In contrast to ganglia, DLN HSV-specific murine CD4 (Zhao *et al.*, 2003) and CD8 (Andersen *et al.*, 2000) T-cells decline dramatically in after recovery from peripheral HSV inoculation, as expected for classic cellular immune responses.

The functional importance of CD8 T-cells in mice is somewhat dependent on the details of the experimental model. Mice deficient in β2-microglobulin, and therefore in CD8 T-cells, have decreased containment of HSV infection in some models (Holterman *et al.*, 1999; Manickan and Rouse, 1995). However, these mice also lack CD1d-restricted NKT cells. Deletion of ICP47 from HSV-1 can decrease neurovirulence, consistent with an effect due to increased recognition of infected cells by CD8 T-cells (Goldsmith *et al.*, 1998). CD8 T-cells alone can provide protection against HSV-1. This was established by immunizing MHC-suitable mice with the $gB1_{498–505}$ epitope, albeit in a specialized vaccinia format (Blaney *et al.*, 1998). The immunodominance of $gB1_{498–505}$ in H-2^b mice was recently exploited to study the relationship between T-cell avidity and diversity and functional protection (Messaoudi *et al.*, 2002). Mice with an allelic variant of H-2^b were found to be relatively resistant to acute HSV-1 lethal infection compared to wild-type H-2^b congenics. The resistant mice had a higher diversity of $gB1_{498-505}$-specific CD8 T-cells in their repertoire, which included cells with very high avidity for peptide-MHC, compared to the wild-type H-2^b mice. The cause of this diversity was hypothesized to be differences in thymic T-cell positive selection, prior to viral infection. Diversity in HLA class I alleles, and hence of the T-cell repertoire, gives a selective advantage in human HIV-1 infection (Trachtenberg *et al.*, 2003) but has not yet been studied for HSV. Contributions to TCR repertoire variability from previous viral infections (Brehm *et al.*, 2002), allelic variations at non-restricting self MHC (Burrows *et al.*, 1995), and minor histocompatibility (Roopenian *et al.*, 2002) loci are well documented and are likely applicable to HSV immunology.

Human CD8 and other immune responses may be expected to differ from murine responses. Lytic replication occurs intermittently in essentially all infected people (Wald *et al.*, 2000), perhaps maintaining or "maturing" responses. Some immune evasion mechanisms, such as transporter of antigen processing (TAP) inhibition by ICP47, are much stronger in humans (Tomazin *et al.*, 1998), potentially influencing CD8 responses. On the balance, HSV stimulates readily detectable CD8 responses in humans. Based on limited tetramer analyses of chronically infected persons, responses are lower than those seen for CMV and EBV (Koelle *et al.*, 2002b; Barouch and Letvin, 2001). HSV infects mainly non-professional APC in a relatively small tissue volume. Some other chronic viral infections such as HPV, HCV, and HBV stimulate even lower-abundance CD8 responses as assessed in the blood. The reasons for this variability remain unknown.

Data concerning the importance of CD8 responses in humans is indirect. The frequency ("pCTL") of circulating CD8+ cells which, in response to HSV-2 antigen, give rise to progeny which kill autologous infected cells in classical limiting dilution assays, is on the order of 1 in 6000 peripheral blood mononuclear cells (PBMC) (perhaps 1 in 1500 or so CD8 T-cells) (Posavad *et al.*, 1996). Among HIV and HSV-2 co-infected persons, the pCTL was inversely correlated with HSV-2 disease severity (Posavad *et al.*, 1997). HLA correlation studies support a functional role for CD8 T-cells as some have shown associations between HLA class I alleles and HSV-2 infection or severity (Lekstrom-Himes *et al.*, 1999). CD8 T-cells with HSV-specific CTL and IFN-γ secretion localize to recurrent genital HSV-2 lesions, and the infiltration of CTL is temporally correlated with clearance of culturable virus (Koelle *et al.*, 1998b). It is not clear if this is simply a reactive "mop-up brigade," or if similar cells are capable of holding HSV replication below clinical threshold or even below the threshold of subclinical shedding (Wald *et al.*, 2000).

HSV-specific CD8 T-cells likely interact with many APC in vivo, including DC, ganglionic cells, and infected cells in the periphery, with different outcomes. The type and condition of APC used in vitro to characterize CD8 responses is also critically important. Fibroblasts are more susceptible than B cells to HSV-mediated down-regulation of HLA class I (Koelle *et al.*, 1993) and poorly re-stimulate memory CD8 responses (Yasukawa *et al.*, 1989; Tigges *et al.*, 1992). Recognition of fibroblasts can be obtained, but knock-out of one or both the immune evasion-related genes, *US12* and *vhs*, or pre-treatment with IFN-γ, is required (Koelle *et al.*, 2001; Tigges *et al.*, 1996). Similarly, recognition of keratinocytes, representative of the likely in vivo target cell in the periphery, requires IFN-γ and specific infection conditions (Mikloska *et al.*, 1996, 2001; Koelle *et al.*, 2001). In vivo, HSV lesions are rich in IFN-γ and display signs of local IFN-γ effects (up-regulation of keratinocyte HLA class II) quite early on (Cunningham *et al.*, 1985; Koelle and Corey, 1995), so these systems may be somewhat physiological. EBV-transformed B cells are more frequently used as APC

in the readout phase of cytotoxicity assays, but their in vivo relevance is questionable. Human HSV-specific CD8 CTL can recognize and kill bystander CD8 T-cells (Raftery *et al.*, 1999), but it is not clear if T-cells are a physiologically important target in vivo.

The number and functional characteristics of human HSV-specific CD8 cells are of interest given their potential varied roles in the nervous system and periphery. Assays of IFN-γ secretion by circulating CD8+ cells in response to HSV-2-infected DC reveals responses (median 0.64%) higher than pCTL estimates (Posavad *et al.*, 2003). While tetramer staining can reveal populations of up to 0.6% of circulating CD8+ cells to be HSV-2-specific (Koelle *et al.*, 2002b), it is not known how many of these cells have CTL, IFN-γ, or both activities. CD8 clones with CTL activity have each displayed specific IFN-γ secretion (Koelle *et al.*, 2001, 2002b). The cytolytic pathways used by HSV-specific CD8 cells are unknown, and are relevant given HSV inhibition of CTL-induced apoptosis (Cartier *et al.*, 2003). Reductions in viral output after interaction with infected target cells, noted for HSV-specific CD4 and NK cells (Yasukawa and Kobayashi, 1985), and for virus-specific CD8 cells in other systems (Yang *et al.*, 2003), are little studied for HSV and CD8 cells. Circulating HSV-2-specific CD8 cells specific for one epitope in virion protein 22 (VP22) are largely CD28+, consistent with a capacity for self-renewal through co-stimulation (Hamann *et al.*, 1997). In other human viral infections, CD28 can vary from epitope to epitope (Koelle *et al.*, 2001). Comparisons by epitope are not yet available for HSV, but we were able to recover HSV-2-specific CD8 CTL of diverse fine specificity using expression of CD28 as a selection criteria (Koelle and Corey, 2003). HSV-specific CD8 cells detected during chronic infection also express CD62L and CCR7 (+), molecules thought to assist in homing to lymph nodes (Sallusto *et al.*, 1999).

The first human CD8 epitopes in HSV were found several years ago, in gB2 and gD2, using a small panel of recombinant vaccinia viruses (Tigges *et al.*, 1992, 1996). Additional HSV antigens recognized by human CD8 T-cells have recently been described with methods such as ORF-spanning peptide pools (Hosken *et al.*, 2006) and expression cloning using whole HSV genome libraries (Koelle *et al.*, 2001, 2002a, 2003). HSV-2 proteins from diverse structural and kinetic classes are now known to be CD8 targets. Known human HSV-1 epitopes are limited to two cross-reactive epitopes in gB and VP13/14 (Tigges *et al.*, 1996; Koelle, 2003). CD8 antigens include, for HSV-2, glycoproteins gB, gD, and gE, the capsid or scaffold protein products of the *UL26* (or in-frame overlapping *UL26.5*) and *UL25* genes, and the tegument proteins VP16 (*UL48*), VP22 (*UL49*), VP13/14 (*UL47*), and the *UL7* gene product. The immediate early non-structural proteins ICP0, ICP4, and ICP27 are also recognized (Mikloska *et al.*, 2001; Koelle, 2003; Koelle *et al.*, 2001, 2002b, 1992; Tigges *et al.*, 1996). IFN-γ ELISPOT surveys have revealed stimulatory peptides in many other ORFS (Hosken *et al.*, 2006) and await complementary studies such as CTL assays and effector cell enrichment from PBMC.

Functional data from longitudinal biopsy studies of genital HSV-2 lesions in humans, in which serial specimens are used for culture of skin-infiltrating lymphocytes, have also been used to probe the CD8 response. HSV-2-specific CD8 T-cells were shown to be dramatically locally enriched, compared to the blood (Koelle *et al.*, 1998b). The mechanism of homing to skin was investigated with HSV-specific tetramers and antibodies to a skin homing-associated molecule, CLA (cutaneous lymphocyte-associated antigen) (Fig. 34.1). Pronounced expression of CLA was detected on HSV-specific cells; expression by control CD8 cells specific for the non skin-associated pathogens EBV and CMV was low (Koelle *et al.*, 2002b). CLA expression enables one-step enrichment of HSV-2-reactive CD8 CTL from blood (Koelle, 2003). Both CLA-positive cells and E-selectin up-regulation are present in human genital HSV-2 lesions (Koelle *et al.*, 2002b). The pathway by which naïve HSV-specific CD8 cells become "programmed" to express CLA is unknown. The cytokines IL-12, TGF-β, and class I IFN are present in HSV lesions (Van Voorhis *et al.*, 1996; Kokuba *et al.*, 1999; Overall *et al.*, 1981), and can synergize with T-cell stimulation through TCR to enhance CLA expression. Plasmacytoid DC can prime CLA on human CD8 T-cells (Salio *et al.*, 2003) and react with HSV as discussed below, but have yet to be documented in HSV lesions.

A CLA-associated determinant binds to E-selectin, a lumenal venular endothelial adhesion molecule up-regulated in the inflamed skin and genital tract (Johansson *et al.*, 1999). Known up-regulators of E-selectin, IL-1, IFN-γ and TNF-α, are enriched in HSV lesions (Doukas and Pober, 1990; Xia *et al.*, 1998; Cunningham *et al.*, 1985; Keadle *et al.*, 2000). In immune mice, treatment with anti-IFN-γ decreases recruitment of lymphocytes adherence to endothelium after vaginal HSV challenge (Parr and Parr, 2003), possibly via decreases in adhesion molecule expression. Parr and Parr documented up-regulation of ICAM-1 and VCAM-1, but not E-selectin or MadCAM-1 (A_EB_7 integrin ligand) in the HSV-2 infected mouse vagina (Parr and Parr, 2000).

Note was made above of the immunodominance of CD8 responses to a specific peptide in H-2^b haplotype mice. There is little information concerning diversity and dominance within the CD8 response in individual HSV-infected

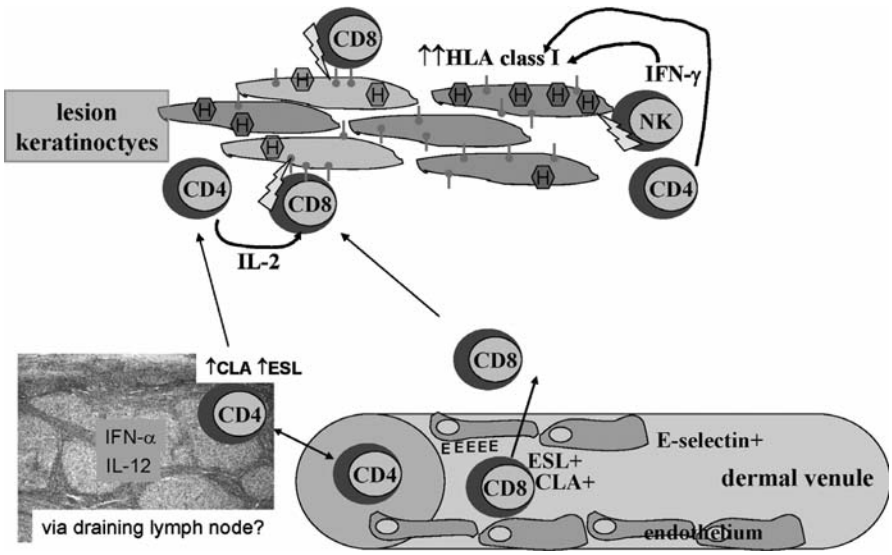

Fig. 34.1. Model of human recurrent genital HSV-2 lesion.

persons. Recently, CLA expression was used to derive panels of HSV-2-specific CD8 CTL clones from blood samples, without secondary in vitro re-stimulation with antigen. The fine specificity of each resultant clone was determined. For most subjects, clonal enumeration detected responses that were focused on one to three HSV-2 peptides per person (Koelle, 2003). Likely, subdominant responses were missed. Similar to other viral systems in humans (Betts *et al.*, 2000), the presence of a specific HLA class I allele did not exert a dominant effect over the selection of viral epitopes for recognition. In some individuals with prevalent alleles such as HLA A*0201 or B*0702, clones restricted by these alleles predominated, while the major response "ignored" these alleles in other persons. Tegument-derived peptides were recognized the most frequently when clonal specificities were categorized by viral structural and kinetic class. Virion input tegument proteins to be recognized in infected APC without a requirement for de novo protein synthesis (Koelle *et al.*, 2001), but it is not known if this contributes to the relatively frequent recovery of clones specific for tegument proteins.

Higher estimates of the diversity of the human CD8 response to HSV-2 have recently been obtained using overlapping peptide sets covering about half of the proteome and IFN-γ secretion as the readout. The median number of ORFs recognized per person was 11. Tegument and immediate early targets of the CD8 response were particularly well represented. The cumulative frequency of IFN-γ producing cells was generally well under 1% of CD8 cells (Hosken *et al.*, 2006). As with the tetramer readouts mentioned above, peptide interrogation of IFN-γ secreting cells show that the integrated CD8 response to HSV-2 in the PBMC compartment is likely much smaller than the response to CMV (Sylwester *et al.*, 2005).

CD4 T-cell responses to HSV

CD4 T-cells have several potential antiviral functions including B-cell help (Yasukawa *et al.*, 1988), CD8 help (Lu *et al.*, 2000), secretion of antiviral (Wong and Goeddel, 1986) and immune-enhancing cytokines such as IFN-γ and TNF-α, and direct cytotoxicity (Yasukawa and Zarling, 1984). These cells localize by day two to recurrent human genital HSV-2 lesions (Koelle *et al.*, 1998b), and also to the uterine cervix (Koelle *et al.*, 2000b) and to the cornea and retina in human ocular HSV infections (Verjans *et al.*, 1996, 1998; Koelle *et al.*, 2000a). Brisk lymphoproliferative responses by PBMC to killed whole HSV reflect a fairly high frequency of HSV-specific CD4 cells in seropositive persons. Quantitative estimates range from about 0.1% of PBMC by limiting dilution proliferation assays (Posavad *et al.*, 1997) to 0.2%–0.4% of circulating CD4+ lymphocytes by IFN-γ intracellular cytokine cytometry (ICC) (Asanuma *et al.*, 2000), and TNFβ ELISPOT (Schmid *et al.*, 1997). CD40L up-regulation, a vital step in B-cell and CD8 T-cell help via DC conditioning, is also detectable on about 0.5% of peripheral blood CD4 cells in response to HSV-2 antigen (Gonzalez *et al.*, 2005). The responder estimates for a specific peptide epitope are quite low (0.002% of CD4

cells) (Kwok *et al.*, 2000). Data are limited, but the response appears to be diverse within infected persons: a median of four HSV-2 ORFs were recognized among seven ORFs tested (Koelle *et al.*, 2000c). The CD4 response coexists with CD4-directed immune evasion strategies (Lewandowski *et al.*, 1993; Barcy and Corey, 2001; Neumann *et al.*, 2003; Trgovcich *et al.*, 2002), covered in detail elsewhere in this volume.

CD4+ cells contribute to protection against HSV in mice. In a zosteriform model, passive transfer of immune CD4+ cells was sufficient to confer protection, and ablation of CD4 cells increased susceptibility in naïve animals (Manickan and Rouse, 1995; Manickan *et al.*, 1995a, b). The CD4 effect was mouse strain-dependent. In the murine vaginal HSV-2 model, recently reviewed (Parr and Parr, 2003), progestin treatment thins the epithelium, increases the susceptibility of naïve animals, and alters local immune responses to HSV-2 (Kaushic *et al.*, 2003). Attenuated (*tk* mutant) intravaginal inoculation is non-fatal, and results in high-level, sterilizing, protective immunity against challenge with wild-type virus. In this model, depletion of CD4 T-cells delayed viral clearance after challenge (Milligan *et al.*, 1998). Qualitative parameters in the murine CD4 response generally support the importance of Th1-like IFN-γ responses. A survey of "long" gD1 peptides predicted to be antigenic by a computer algorithm revealed distinct elicitation of Th1 (IFN-γ-high IL-4 low) and Th2 (IFN-γ-low IL-4 high) responses by individual peptides given with an oil-in-water adjuvant. The Th1 peptides were more protective against an ocular HSV-1 challenge than were the Th2 peptides, and protection was mediated by CD4+ cells (BenMohamed *et al.*, 2003).

Recently, a subset of CD4+ T-cells with constituitive expression of CD25 (T_{reg}) have been described that have suppressor or regulatory activity (Chatenoud *et al.*, 2001). Depletion of T_{reg} in mice led to increased CD8 CTL responses against the HSV $gB1_{498–505}$ epitope, which persisted into the memory phase after T_{reg} recovery (Suvas *et al.*, 2003). CD25 depletion lead to a short window period of faster viral clearance from the inoculation site, while adoptive transfer of CD4+CD25+ cells (from naïve mice) led to delayed clearance. HSV-1 infection of mice increased the in vitro, per-cell suppressor activity of their CD4+CD25+ splenocytes. Interestingly, CD25+ cell depletion worsened CD4 T-cell mediated corneal immunopathology in a mouse model of HSV keratitis, even though it sped viral clearance (Suvas *et al.*, 2004). Recently, blood CD4+ $CD25^{high}$ cells have been shown to suppress recall lymphoproliferative responses to HSV-2 antigen in humans (Diaz and Koelle, 2006). While T_{reg} have functional TCR, they often act in an antigen non-specific manner and it is not known if HSV-specific T_{reg} occur in mice or humans.

It is not yet clear if CD4 responder cell numbers or function correlate with disease severity in humans. A general relationship is present between lower overall CD4 cell numbers and HSV shedding among HIV-infected men (Schacker *et al.*, 1998). However, in a cross-sectional study of HSV-2/HIV-1 co-infected men, HSV-2 severity did not correlate with HSV-2-specific CD4 cell numbers (Posavad *et al.*, 1997). Data from small numbers of subjects indicate that proliferative responses to specific viral proteins may be higher in symptomatic than asymptomatic persons (Spatz *et al.*, 2000). Responses to whole virus may be lower in symptomatic than in asymptomatic persons (reaching significance on one study (Frenkel *et al.*, 1989) and trending to significance in a small study (Singh, 2003a). Studies of HSV-1 disease severity and IFN-γ responses (likely, but not proven, to be CD4 mediated) were close to (Spruance *et al.*, 1995) or achieved (McKenna *et al.*, 2001) statistical significance for association between higher responses and milder disease. In a small study of HSV-2 infected persons with proliferative responses to HSV antigen were measured, asymptomatic seropositives had higher IFN-γ and lower IL-10 responses to HSV than did persons with symptomatic disease (Singh, 2003a). Longitudinal studies revealed a trend to decreased PBMC IFN-γ responses to HSV antigen over time after episodic acyclovir therapy of a genital herpes outbreak (Singh, 2003a). Two recent vaccine trials (Corey *et al.*, 1999; Stanberry *et al.*, 2002), used similar purified recombinant HSV-2 protein antigens, but different adjuvants. Partial clinical efficacy was reported only for the vaccine that included an adjuvant (alum and 3d-MPL) that elicits Th1-like CD4 responses, although head-to-head comparisons are not possible. In the recent human vaccine experience, elicitation of CD4 (and antibody) responses has not provided high-level protection from genital HSV-2 infection or disease (Koelle and Corey, 2003).

HSV-specific CD4 clones have diverse functional activities in vitro. They are heterogeneous with regards to cytotoxicity towards HSV-2-infected B-cells. All blood and genital lesion-derived CD4 clones have cytolytic potential, as all display cytotoxicity when maximally stimulated by peptide-pulsed B cells (Koelle *et al.*, 1998a; 2000a). We can speculate that CD4 immune evasion mechanisms differentially modulate the killing of infected cells by HSV-specific CD4 clones; differential resistance to CD4 CTL-induced apoptosis has been demonstrated (Jerome *et al.*, 1998). Cornea-derived HSV-1-reactive CD4 clones, in contrast, have not had CTL activity even when maximally stimulated (Koelle *et al.*, 2000a). Killing of infected keratinocytes is detectable if they are pre-treated with IFN-γ (Mikloska *et al.*, 1996). For blood-derived CD4 clones, the cytolytic mechanism for B cell targets appears to involve granules and not the Fas/FasL pathway (Yasukawa *et al.*, 1999).

The CD4 response to HSV is broadly directed. Published targets are non-structural proteins ICP8 and the *UL50* product dUTPase, glycoproteins gB, gC, gD, gE, and gH, tegument proteins VP11/12, VP13/14, VP16, VP22, and the *UL21* gene product, and the capsid protein VP5 (Koelle and Corey, 2003). Responses to the *UL29* gene product, a DNA binding protein, were detected in the blood of an HSV-2-exposed, but HSV-1/HSV-2 seronegative person and possibly represent sensitization without seroconversion (Posavad *et al.*, 2003). The HLA DR, DP and DQ loci are each well represented among alleles restricting peptide-specific responses. The population prevalence of responses to the candidate vaccine compounds gB2 and gD2 is quite high, over 80%, among HSV-2 seropositives. Responses to some of the tegument proteins can match these levels (Koelle *et al.*, 2000c).

T-cells are required in the immunopathogensis of experimental herpes simplex interstitial keratitis (HSK) in mice (Metcalf *et al.*, 1979). Controversy surrounds the requirement for HSV-specific CD4 T-cells in the pathogenesis of this disease. In humans, tissue from end-stage lesions, namely, corneas removed for blinding HSK have been examined for the presence of HSV-specific T-cells. Two research groups are in agreement (Verjans *et al.*, 1998; Koelle *et al.*, 2000a) that HSV-specific, HLA class II-restricted, CD4+ T-cell clones are readily established from these specimens. These cells recognize HSV-1/HSV-2 cross-reactive tegument protein epitopes in some instances, and generally have a Th1-like cytokine profile. A minority of patients with more superficial corneal HSV infections progress to HSK. The effects of potent immunotherapy directed at genital herpes on the natural history of ocular HSV infections deserve close medical attention.

T-cell costimulation and HSV

Both CD4 and CD8 T-cells require costimulation during primary, and to a lesser extent, recall, responses. The two-signal model of T-cell activation holds that in addition to an agonistic ligand of the T-cell receptor, usually MHC plus peptide, one or more additional signals instruct a T-cell to become activated. Major co-stimulatory pathways include CD80 and CD86 on APC interacting with CD28 on T-cells, and CD40 on APC interacting with CD154 on T-cells. In vivo, a complex mixture of activatory and inhibitory stimuli are integrated by signal transduction pathways.

The CD40-CD40L system is required for immunoglobulin class switching and the development of Th1 CD4 responses. One human with a mutation in CD40 has been found to be hypersusceptible to HSV disease (Fuleihan, 2001; Garcia-Perez, 2003). Increased monocyte CD80 and CD86 expression after incubation of immune human PBMC with HSV is not inhibited by anti-IFN-γ, and may be due to CD40L conditioning of monocytes (Singh, 2003b). Deletion of CD40L or administration of anti-CD40L antibody decreased survival after HSV-1 footpad or vaginal inoculation, and delayed DLN CD4 responses to HSV antigen (Edelmann and Wilson, 2001; Inagaki-Ohara *et al.*, 2002). Enhancement of CD40L expression via an exogenous plasmid co-administered with a gD2 plasmid vaccine increased splenocyte memory Th1 responses to gD2 protein, and also increased survival in a murine HSV-2 challenge model (Sin *et al.*, 2001). As CD40L induces B cell maturation, the increased survival after HSV-1 challenge of mice with GVHD associated with CD40L protein administration was ascribed to enhanced anti-HSV antibodies (Beland *et al.*, 1998).

CD40 and CD40L are each members of the large TNF receptor and TNF families, respectively (Croft, 2003). Other members of these families have also been implicated in HSV pathogenesis. Lopez observed in 1975 that Balb/c mice were more susceptible than C57BL/6 mice to mortality after intraperitoneal infection with HSV-1 (Lopez, 1975). The exact genetic basis of this difference remains unknown, but at least one prominent locus has been mapped to a region of murine chromosome 6 which contains TNF receptor genes (Lundberg *et al.*, 2003). CD137 (4–1BB) is a TNF family member expressed by T-cells. CD137 provides a positive co-stimulatory signal which can augment anti-viral immunity (Halstead *et al.*, 2002). Mice deficient in CD137 or treated with anti-CD137L mAb show less corneal pathology in a HSV keratitis model (Seo *et al.*, 2003). HVEM, a member of the TNFR family, is best known as a receptor for HSV entry. Less is known concerning the potential for immune modulation by gD ligation of HVEM, which is expressed by many APC and lymphocytes (La *et al.*, 2002).

An expanding family of membrane proteins in the B7 family (Prasad *et al.*, 2003) expressed by APC and stromal cells have complex positive and negative effects on members of the CD28 family (Rudd and Schneider, 2003) expressed on T-cells. CD80 or CD86, when separately coadministered with gD2 as plasmids in mice, had adjuvant and suppressive effects, respectively, using immunologic and clinical end-points (Flo *et al.*, 2000, 2001). In a vaginal model of HSV-2 infection, mice deficient in both CD80 and CD86 failed to control the infection (Thebeau and Morrison, 2002). Immune correlates included decreases in CTL responses, local IFN-γ secreting T-cells, and CD40L up-regulation by CD4 T-cells in response to antigen, and alterations in HSV-specific immunoglobulin isotypes (Thebeau and Morrison, 2003). Systemic interruption of

B7 family/CD28 family interactions with CTLA4-Ig fusion ameliorates the course of HSK in mice, correlating with decreased memory CD4 responses to HSV (Gangappa *et al.*, 1998). Local blockade of B7–1 and B7–2 (CD80 and CD86), which are present on corneal LC, also prevents HSK in mice (Chen and Hendricks, 1998). Thus far, few data link the B7 pathway and HSV in humans. A small study found that monocytes from HSV-infected persons displayed impaired CD80 and CD86 up-regulation in response to exogenous IFN-γ (Singh, 2003a).

Antibody responses to HSV

Antibody responses to HSV are known to be functionally important in the context of neonatal infection. The risk of clinically significant infection after delivery through a HSV culture-positive cervicovaginal tract is much higher during primary than during recurrent maternal infection (Brown *et al.*, 2003). In the latter instance, maternal IgG antibodies are transferred to the neonate across the placenta. Antibodies cooperate with neutrophils, monocytes, and NK-like cells in mediating antibody-dependent cell-mediated cytotoxicity (ADCC) (Kohl, 1991). Of interest, recent candidate vaccines with limited efficacy stimulated low ADCC titers, despite eliciting high levels of binding and neutralizing antibody (Kohl *et al.*, 2000).

Local antibody responses are of interest in the female genital tract. In the murine vaginal HSV-2 model, mice are protected by non-lethal vaginal infection with a *tk*- HSV-2 strain (Parr and Parr, 2003). Antibody plays a significant role in early virus containment after vaginal challenge, as shown with B-cell knock-out mice (Parr and Parr, 2003). Little evidence for local secretory IgA as an effector molecule was found, similar to findings in human genital secretions (Boggess *et al.*, 1997). In contrast, local IgG with neutralizing activity was readily detected. Attempts to induce vaginal immunity in mice through a "common mucosal immune system" pathway, via intranasal or oral immunization, have been successful in some models, but protection has not been mechanistically linked with local IgA. Human IgG immunoblot kinetic patterns differ between serum and cervical samples, suggesting that genital tract IgG may reflect local synthesis in addition to diffusion of plasma protein (Ashley *et al.*, 1994). Similar data has been presented for local antibody synthesis in HSV infections of the eye (Peek *et al.*, 2002) and CNS, especially late into HSV encephalitis (Sauerbrei *et al.*, 2000). Vaginal tissues from vaginally immunized mice contain many fairly persistent IgG-producing plasma cells, while similar vaccinations at other sites do not lead to this cell localization (Parr and Parr, 2003). Recently, specific expression of the mucosal homing molecule A_4B_7 integrin (MLA, CD103) has been demonstrated for B-cells specific for a gastrointestinal pathogen (Gonzalez *et al.*, 2003). Similar B-cell homing to the genital tract is rational but has not yet been demonstrated.

The development of a specific immune evasion function for antibodies is additional evidence of the functional importance of the antibody response (Nagashunmugam *et al.*, 1998). The HSV-encoded gE-gI high-affinity FcγR receptor is expressed on infected cells. gE-gI binds human, but not mouse, Fcγ, so a murine zosteriform spread model combines injection of human immune serum and challenge with wild-type or gE knockout HSV-1. The gE knockout has decreased virulence in the context of immune but not pre-immune human serum treatment (Lubinski *et al.*, 2002), consistent with an effect on pathogenesis mediated through immune escape. Recently, a region of gE1 capable of eliciting blocking antibodies that reduce Fcγ binding was identified (Lin *et al.*, 2003), and it has been proposed that inclusion of such an immunogen could assist vaccination against HSV (Lin *et al.*, 2004).

Neutralization of clinical HSV isolates by rabbit antisera to HSV-1 vs. HSV-2 formed the basis for early HSV typing methods. Antibodies that inhibit the absorption, or neutralize, HSV *in vitro* are specific for gB, gC, gD, or gH/gL (Fuller *et al.*, 1989; Fuller and Spear, 1987, 1985; Sanchez-Pescador *et al.*, 1992). Antibodies to gB or gD inhibit neuron to keratinocyte spread in a two-cell model (Mikloska *et al.*, 1999). Passive transfer of antibodies specific for several of these glycoproteins is protective in animals (Su *et al.*, 1996; Balachandran *et al.*, 1982); in some cases, ADCC may be involved rather than classic neutralization (Kohl *et al.*, 1986). Recent advances in the study of HSV receptors, particularly for gD, have provided a structural basis for the differential neutralization of various gD-specific mAb (Whitbeck *et al.*, 1999). Local application of neutralizing mAb can prevent disease after vaginal inoculation (Whaley *et al.*, 1994). As reviewed elsewhere (Koelle and Corey, 2003), vaccines eliciting neutralizing titers above those seen in natural infection have not been clinically effective.

Innate immunity

The importance of innate immunity is readily demonstrable in primary infection and in acute lethality models. HSV immune escape mechanisms that target innate complement and class I interferon responses are reviewed elsewhere in this volume. Mobile leukocytes and tissue stromal cells collaborate in innate immunity. As reviewed

at the outset of this chapter, dendritic cells form a crucial bridge between innate and adaptive immunity. Distinctions between innate and acquired are becoming increasingly arbitrary, as specialized pattern recognition receptors such as toll-like receptors (TLR) and NK-cell activatory receptors that recognize viral proteins in highly specific fashions (Arase *et al.*, 2002) are being described.

Complement is another major bridge between innate and acquired immunity (Mullick *et al.*, 2003). As discussed elsewhere in this volume, HSV gC specifically binds to and inactivates certain complement components (Lubinski *et al.*, 2002). Elegant studies in complement-deficient mice have shown through genetic "complementation" that gC is a virulence factor in intact animals (Lubinski *et al.*, 1999). Clinically, persons with complement deficiencies are not generally felt to have more serious HSV infections than the general population (Walport, 2001). This can be interpreted in two ways: either complement is already maximally disabled, or complement-dependent mechanisms, while active, are redundant in the otherwise normal host.

Neutrophils are not typically considered major antiviral effector cells. Neutropenic patients can have severe HSV infections, but usually in settings in which lymphocyte number or function are also impaired. Isolated neutropenia, or disorders of phagocyte effector functions such as chronic granulomatous disease (CGD), are not generally clinically associated with severe HSV infections (Andrews and Sullivan, 2003). Neutrophils do strongly infiltrate genital HSV lesions in humans at the pustule stage. Giemsa stains of cytospin preps of pustule cells from day 2–3 HSV-2 buttock or thigh lesions show >95% neutrophils (D. M. Koelle *et al.*, unpublished data). HSV vesicle fluid is rich in chemokines that can attract neutrophils (Mikloska *et al.*, 1998), and mice with genetic knock-out of chemokines which attract neutrophils have alterations in HSV pathogenesis (Tumpey *et al.*, 1998). IL-17 can assist in chemokine synthesis, is up-regulated in infected human corneas, and made by keratocytes in response to HSV infection (Maertzdorf *et al.*, 2002). Neutrophils localize to the HSV-2-infected vagina in mice. Depletion of neutrophils has measurable effects on viral clearance in both primary and vaccination/challenge models, especially at early time points and locally in the vagina (Milligan, 1999; Milligan *et al.*, 2001). Neutrophil antiviral effector functions are not well characterized. CGD neutrophils, which have defects in the formation of reactive oxygen species, digest HSV normally (Van Strijp *et al.*, 1990). Neutrophils have ADCC activity against infected cells (Siebens *et al.*, 1979). Some defensins that are expressed by neutrophils have potent anti-HSV activity (Sinha *et al.*, 2003). Neutrophils matrix metalloproteinases have been implicated in tissue damage related to HSV infection (Lee *et al.*, 2002b).

Many cells respond to viral infection by the production of class I interferons. Protein products of this multigene family (interferon alphas and beta) have autocrine and paracrine activity and trigger the expression of a characteristic pattern of genes (interferon-stimulated genes, ISG). Class I interferons have pronounced anti-HSV activity in vitro and in animals (Connell *et al.*, 1985) and appear to also be active in humans (Lebwohl *et al.*, 1992). Limitin is a class I interferon-related protein with anti-HSV activity in an acute murine HSV-1 lethality model (Kawamoto *et al.*, 2003) and potentially less toxicity.

The ISG pattern has been detected with expression arrays after infection of several cell types with HSV. Deletion of immediate early genes has been helpful to unmask this response (Nicholl *et al.*, 2000). The canonical class I interferon signaling pathway through TyK2, JAK1, and STAT1 is not required for ISG up-regulation. Therefore, an autocrine class I interferon effect is unlikely be responsible for ISG activation. HSV virions, or VP16-mutated HSV which cannot replicate in fibroblasts, also elicits ISG expression without eliciting an interferon biological activity. Cells may have an alternative sensor mechanism to trigger the ISG pathway (Mossman *et al.*, 2001). If VP16 is restored, ISG up-regulation is not detected, while cycloheximide restores ISG mRNA up-regulation during infection with wild-type HSV. HSV may therefore direct the synthesis of protein(s) that block ISG transcription, with some data suggesting ICP0 can mediate this function (Eidson *et al.*, 2002; Harle *et al.*, 2002). HSV evasion of class I interferon through γ34.5 is discussed elsewhere in this volume and was recently reviewed (Leib, 2002). In brief, γ34.5 antagonizes the protein kinase R (PKR) effector pathway for class I interferons. While PKR phosphorylates, and inactivates, EIF2α, thus reducing viral protein synthesis, γ34.5 has or induces a phosphatase activity which removes this block. Deletion of γ34.5 reduces the virulence of HSV by several orders of magnitude (Chou *et al.*, 1990), an effect that disappears in IFN-α receptor or PKR knock-out, but not RNAse-L knock-out, mice (Leib *et al.*, 1999; 2000).

Interferon-alpha is confusingly known as leukocyte interferon, in contrast to interferon-gamma, which is made more or less exclusively by lymphocytes (which are leukocytes). It was recognized very early that PBMC exposed to HSV make large amounts of interferon-alpha, regardless of donor HSV serostatus (Haahr *et al.*, 1976). Rare leukocytes in PBMC that respond to HSV by producing IFN-α have been termed natural interferon producing cells (NIPC). A prominent NIPC was recently identified as dendritic cell subtype termed plasmacytoid dendritic cells or

pDC (Siegal *et al.*, 1999; Colonna *et al.*, 2002; Fitzgerald-Bocarsly, 2002). These rare (<1% of PBMC) cells produce enormous amounts of IFN-α on a per-cell basis by up-regulating IFN mRNA after stimulation. The phenotype of pDC varies slightly from humans (CD4+ CD11c−) to mice (CD11c+CD11b-Gr-1+B220+). pDC recognize many DNA and RNA viruses, produce several different IFN-α gene products and IFN-β, and are decreased in advanced HIV infection (Chehimi *et al.*, 2002), a condition characterized by susceptibility to viral infections. Their selective expression of TLR7 (Hornung *et al.*, 2002) makes them a logical target for imidazoquinolines, TLR7 agonists (Hemmi *et al.*, 2002) with anti-HSV activity (Spruance *et al.*, 2001). It is not yet known if pDC localize to HSV-2 lesions, but pDC-like cells express CLA, a skin-homing molecule (Schmitt *et al.*, 2000), and localize to melanoma lesions (Salio *et al.*, 2003) and some inflammatory skin conditions (Wollenberg *et al.*, 2002).

Clearly, several cell types in PBMC are capable of innate responses to viral stimuli. For example, monocytes, while secreting less IFN-α than pDC on a per-cell basis (Fitzgerald-Bocarsly, 2002), are more abundant in the blood and are the main NIPC for Sendai virus (Feldman *et al.*, 1994). Dendritic cell plasticity allows non-pDC to make large amounts of IFN-α in response to some viruses (Diebold *et al.*, 2003). Monocyte-lineage dendritic cells (derived in vitro with GM-CSF and maturation stimuli) also produce IFN-α in response to HSV (Rong *et al.*, 2003). Other monocyte pro-inflammatory responses to HSV are discussed below.

The viral factor(s) involved in the detection of HSV by the innate immune system have been incompletely defined and may vary between different host responses and responder cell types. UV-inactivated virus or aldehyde-fixed, HSV-infected cells can stimulate PBMC IFN-α responses, indicating that viral replication may not be required (Rong *et al.*, 2003; Lebon, 1985). Ankel *et al.* (1998) have presented data that glycoprotein D of HSV-1 or HSV-2 is a potent trigger of IFN-α production by human PBMC. Insect cells expressing plasma membrane gD (Sf9-gD1), but not other HSV-1 glycoproteins, or soluble truncated gD1, but not soluble gH/gL complexes, stimulate PBMC IFN-α secretion. Responses to HSV-1 or Sf9-gD1 were inhibited by HSV immune human sera and also monoclonal anti-gD (Fitzgerald-Bocarsly *et al.*, 1991; Ankel *et al.*, 1998). Inter-strain differences in the ability of HSV-1 strains to stimulate various NIPC have been detected (Rong *et al.*, 2003), but their structural or genetic basis is unknown.

The host factors by which cells detect HSV and other viruses are under intense study (Vaidya and Cheng, 2003). TLR2, but not TLR4, has been implicated in sensing HSV-1 in a mouse intraperitoneal challenge model (Wakimoto *et al.*, 2003; Finberg *et al.*, 2003). A study of pDC isolated from bone marrow of gene knock-out mice, using IFN-α production as the readout, suggests a role for TLR9, but not TLR4, for responses to HSV-2 (Lund *et al.*, 2003). Both human and mouse pDC express TLR9 (Kadowaki *et al.*, 2001; Boonstra *et al.*, 2003).

MyD88 knock-out also reduced IFN-α production, but this adaptor molecule is downstream of many receptors (Takeuchi and Akira, 2002). Confirmatory evidence of the TLR9 and MyD88-dependence of pDC IFN-α responses to HSV-1 was obtained using pDC sorted from splenocytes (Krug *et al.*, 2003). HSV replication was not required, as UV-inactivated HSV-1 was able to stimulate IFN-α. In vivo, neither TLR9 knock out (in the Balb/c background) nor MyD88 knock out (in the C57BL/6 background) had any deleterious effect on the local control of HSV-1 infection. Because mice with deficiency of either the class I IFN receptor or PKR fail to contain HSV-1 infection (Luker *et al.*, 2003; Leib *et al.*, 2000), these data suggest that MyD88- and TLR-independent activation of the class I IFN pathway is possible.

Certain unmethylated CpG oligodeoxynucleotide sequences are the primary known ligands for TLR9. The IFN-α response of PBMC is inhibited by the lysosomotropic agent chloroquine, consistent with a requirement for the acidification of endosomes containing the triggering signal (Feldman *et al.*, 1994). Of note, TLR9 has been reported to be primarily expressed intracellularly (Ahmad-Nejad *et al.*, 2002). A possible link between endocytosis and TLR9 comes from the demonstration that mannose receptors (MR) on DC are involved in NIPC responses (Milone and Fitzgerald-Bocarsly, 1998). Anti-MR sera block NIPC responses, and NIPC with characteristics of human pDC (CD4+) express MR. MR is involved in internalizing ligands via endocytosis. If gD is a primary trigger for IFN-α, HSV receptors (Spear *et al.*, 2000), including an MR (Brunetti *et al.*, 1995), that recognize gD are logical candidates. Monoclonal antibodies to galactosyl cerebroside/sulfatide can block IFN-α production by PBMC in response to HSV-1 or gD (Ankel *et al.*, 1998); related structures are present on some HSV receptors (Shukla and Spear, 2001).

The consequences of NIPC interactions with HSV have only begun to be described. In addition to secreting IFN-α, pDC can secrete other cytokines that influence priming such as IL-10 or IL-12 (Krug *et al.*, 2003), prime T-cells (Fonteneau *et al.*, 2003), increase T_{reg} activity ((Gilliet and Liu, 2002), or be tolerogenic (Kuwana *et al.*, 2001). PDC priming of pseudo-naïve CD8 cells specific for a melanoma antigen reportedly up-regulated CLA expression (Salio *et al.*, 2003); as noted above, HSV-2-specific CD8 cells express CLA, but thus far animal models have not indicated that pDC are functional APC for HSV-1 (Allan *et al.*,

2003). HSV-2 interaction with pDC leads to secretion of IFN-α and IL-12, which promote the expression of CLA on human memory HSV-2-specific CD4 T-cells in an ex vivo reconstitution system (Koelle *et al.*, 2006). IFN-α biases Th responses to the Th1 phenotype in humans (Rogge *et al.*, 1998). Murine NIPC (Asselin-Paturel *et al.*, 2001) secrete IFN-α in response to HSV, but IFN-α biasing towards Th1 responses is altered in mice (Rogge *et al.*, 1998), limiting the application of murine studies to humans. Other host cytokines can have modulatory effects on NIPC responses to HSV in vitro (Gary-Gouy *et al.*, 2002; Payvandi *et al.*, 1998) and may also influence pDC in vivo.

In addition to IFN-α, monocytes and macrophages secrete other immunomodulatory cytokines after exposure to HSV. TNF-α has anti-HSV activity by synergizing with IFN-γ (Wong and Goeddel, 1986) and up-regulating nitric oxide (Kodukula *et al.*, 1999) and has both protective and immunopathologic roles in various HSV models (Kodukula *et al.*, 1999; Keadle *et al.*, 2000; Croen, 1993; Adler *et al.*, 1997). Murine IFN-γ-primed macrophage-like cells produce TNF-α in response to HSV. UV- or formaldehyde-killed virus, a gL-deficient mutant (unable to enter cells), and a VP16 mutant unable to initiate transcription elicit TNF-α elicit partial TNF-α responses, while purified gD1 is a potent stimulator in this system. Responses to HSV-2 involved transcriptional (NF-κB, ATF/Jun) and translational control (Paludan *et al.*, 2001). The use of gD vaccines (Stanberry *et al.*, 2002) and anti-TNF therapies (Bresnihan and Cunnane, 2003) in humans raises interest in studying HSV-TNF-α biology in the natural host.

IL-12 is a cytokine with prominent effects on T-helper polarization. IL-12 p40 mRNA is increased in human genital HSV-2 lesions (Van Voorhis *et al.*, 1996) and after infection of mice with HSV-1 (Kanangat *et al.*, 1996). In contrast to TNF-α, murine macrophage-like cells had poor IL-12 p40 production in response to UV- or heat-killed HSV-1. Corneal epithelial cells are poor sources of IL-12 in response to HSV when compared to macrophage-lineage cells, but produce an uncharacterized activity that induces IL-12 in macrophages. Infected macrophages are also low producers of IL-12, based on two-color confocal microscopy, indicating that paracrine induction may also take place when macrophages are infected with HSV-1. In contrast to some monokines, viral protein synthesis appears to be required for macrophage IL-12 responses to HSV (Kumaraguru and Rouse, 2002). Detectable IL-12 is present in human HSV-1 vesicle fluid, and fetal and adult keratinocytes synthesize IL-12 in response to live HSV-1 infection (Mikloska *et al.*, 1998).

IL-12 is one of several cytokines with adjuvant activity for HSV vaccines (Lee *et al.*, 2003). In a murine intravaginal HSV-2 model, IL-12 knock-out mice had faster mortality than wild-type controls (Harandi *et al.*, 2001a). IL-12 has anti-angiogenic activity in the cornea, mediated through IFN-γ and IFN-γ up-regulated chemokines, which may be relevant in herpes keratitis (reviewed by Dr. Rouse elsewhere in this volume) (Lee *et al.*, 2002a). IL-18 is an IL-1 family cytokine which synergizes with IL-12 in inducing IFN-γ secretion by T cells. When leukocytes from HSV-naïve mice are stimulated with live HSV-2, roles for IL-12, IL-18, and also IFN-αβ can be demonstrated for IFN-γ secretion (Malmgaard and Paludan, 2003). Exogenous IL-18 improves survival in an acute HSV-1 lethality model, an effect that depended on IFN-γ but not NK cells (Fujioka *et al.*, 1999). Knock-out of IL-18 alone accelerated death in a murine HSV-2 intravaginal acute lethality model. Depletion of IL-18 on an IL-12 deficient background further compromised survival. In the setting of sublethal attenuated HSV-2 vaccination, followed by a vaginal HSV-2 challenge, knock-out of either IL-12, IL-18, or both had no effect on survival, despite alterations in lymphoproliferation and immunoglobulin isotype switching (Harandi *et al.*, 2001a).

IL-15 is an IL-2-related cytokine with profound effects on NK cells. HSV increases IL-15 in vivo in mice (Tsunobuchi *et al.*, 2000) and in human PBMC; HSV-mediated increases in NK activity in these cultures are dependent on IL-15, and IL-15 neutralization increases productive HSV infection (Ahmad *et al.*, 2000). Exogenous IL-15 can be protective in a murine HSV challenge model (Tsunobuchi *et al.*, 2000). IL-15-deficient mice are about 100-fold more susceptible to acute lethality after vaginal HSV-2 inoculation (in the setting of progesterone treatment) than are wild-type congenics (Ashkar and Rosenthal, 2003). IL-15 can also increase acquired responses to HSV in the context of HSV DNA/cytokine cDNA co-vaccination (Sin *et al.*, 1999).

Experimental HSV studies in mice typically use gene disruptions, antibody blockade, or cell depletion to study innate immunity. Fewer studies have addressed the human host and natural infection. CD40 assists priming at an intersection between innate and acquired responses, and as mentioned above, a person with a CD40 mutation and severe HSV has been described. Mutations affecting IFN-γ receptors or IL-12/IL-12R are not uncommon, but no instances of severe viral infection have been reported (Fieschi *et al.*, 2003; Fieschi and Casanova, 2003). Homozygous mutations in the C-terminal phosphorylation domain of STAT1 reduce both IFN-α/β and IFN-γ signal transduction (Dupuis *et al.*, 2003). As in *IFNGR1* (IFN-γ receptor ligand-binding chain), *IFNGR2 (*IFN-γ receptor signaling chain), *IL12B* (IL-12 p40 protein), and *IL12RB1* (IL-12 receptor β1 chain*)* mutations, disseminated bacillus Calmette-Guerin (BCG) infections occur. Disseminated,

fatal childhood infection with HSV-1 is also observed. Cell lines from these subjects are resistant to the anti-HSV effects of IFN-α in vitro (Dupuis *et al.*, 2003). NK cell deficiencies are discussed below. Mutations in UNC-93B, an endoplasmic reticulum protein of unknown function, have recently been associated with lethal HSV infections and impairment of the PBMC IFN-α response to HSV virions (Casrouge *et al.*, 2006).

IL-10 is another monocyte product with pleotropic anti-inflammatory and immune modulatory activities. In models of HSV keratitis, IL-10 administered in a variety of formats decreases interstitial keratitis without increasing viral replication (Daheshia *et al.*, 1997). IL-10 mRNA is locally enriched in genital HSV-2 in humans (Van Voorhis *et al.*, 1996) and protein is detected in areas of murine (Stumpf *et al.*, 2002) and human (Mikloska *et al.*, 1998; Ongkosuwito *et al.*, 1998) HSV-1 infection. The sources of IL-10 in herpes infection in vivo are not known, and could include parenchymal cells (Zak-Prelich *et al.*, 2001) and responding lymphocytes (Koelle *et al.*, 2000a). An association between a homozygous polymorphism in the IL-10 promoter and HSV serostatus (Hurme *et al.*, 2003) awaits confirmation.

Immunomodulators and HSV

Several groups have attempted to modulate HSV infection by altering innate or HSV-specific immune responses. CpG ODN co-administration potentiates the immunogenicity and efficacy (against subsequent vaginal HSV-1 challenge) of vaccination with an immunodominant HSV CD8 peptide (Gierynska *et al.*, 2002). CpG also had an adjuvant effect when given prior to vaccination with an intravaginal sublethal dose of live HSV-2 (Harandi *et al.*, 2003). CpG ODN given intravaginally without an HSV constituent protects against subsequent intravaginal HSV-2 challenge and can also be therapeutic (Harandi *et al.*, 2003; Ashkar *et al.*, 2003; Pyles *et al.*, 2002). IFN-γ and the IFN-γ inducers IL-12 and IL-18 were induced locally by CpG ODN. Knock-out of IL-12 and IL-18 reduced protection in one model, while the effect of IFN-γ knock-out on CpG ODN protection may depend on CpG and virus dosing. Infiltration of NK1.1 (+) NK or NKT cells and CD11b+ cells (likely DC) and proliferative changes in the genital mucosa were associated with CpG ODN but not control ODN administration. The imidazoquinoline resiquimod is a TLR7 agonist that increases local IFN-α levels after topical application (Hemmi *et al.*, 2002; Arany *et al.*, 1999). Unfortunately, a phase III clinical trial of topical resiquimod for recurrent genital HSV-2 in humans was stopped without evidence of clinical activity (February 2003).

Chemokines

Chemokines are a large family of small, basic proteins with diverse cellular sources and biological activities (Bacon *et al.*, 2002). HSV-1 is not known to encode chemokine or chemokine receptor homologues. HSV-1 vesicle fluid from humans contains high levels of CCL5 (RANTES), CCL3 (MIP-1α), and CCL4 (MIP-1β) (Mikloska *et al.*, 1998). Cultured human keratinocytes synthesize these chemokines in response to live HSV-1 infection, despite cytopathic effect. The chemokines CCL2 (MCP-1), CCL3, CCL5, and CXCL8 (IL-8) are significantly increased the cerebrospinal fluid in HSV encephalitis in humans (Rosler *et al.*, 1998).

Chemokines have been hypothesized to assist with recruitment of lymphocytes and monocytes to areas of infection (Mikloska *et al.*, 1998). Studies of HSV infection and vaccination in wild-type and knock-out animals, and in vitro studies, have implicated chemokines and their receptors in adaptive, and pathogenic, host responses. Local CCL5 (RANTES) and CCL2 (MCP-1) levels are increased in the vagina after HSV-2 infection. Innate and acquired immunity both contribution to CCL5 accumulation as accelerated, heightened responses are observed in previously vaccinated animals. For CCL2, in contrast, vaginal levels were decreased after challenge of vaccinated vs. non-vaccinated animals, and local CCL2 was correlated with tissue inflammation and damage. Vaginal CCL5 protein levels were partially dependent on IFN-γ after challenge of vaccinated wild-type vs. IFNγ -/- mice (Harandi *et al.*, 2001b). CCL5 is persistently expressed in latently infected murine ganglia (Halford *et al.*, 1996). Knockout of CCL3 (MIP-1α) (Menten *et al.*, 2002) lessens histologic HSK in mice, correlating with decreased ingress of CD4+ cells and decreased IFN-γ and IL-2 mRNA levels, without effecting HSV-1 clearance (Tumpey *et al.*, 1998). Neutralization of CXCL10 (IP-10) also lessened leukocyte infiltration and pathology while transiently increasing corneal HSV-1 replication after scarification (Carr *et al.*, 2003). As with T_{reg} cells, chemokines can have complex influences on HSV pathogenesis. CXCR3-deficient mice have decreased clearance of HSV-1 in a CNS infection model, but also show increased survival (Wickham *et al.*, 2005).

NK cells

Human NK cells with activity against non HLA-restricted cytotoxicity against HSV-infected fibroblasts have long been described (Lopez *et al.*, 1982). NK activity against HSV-infected fibroblasts was always observed to take many

hours, in contrast to the killing of tumor cells such as K562, and some distinctions were found between the two effector functions. The recognition that IL-12, IL-15, and IFN-α are induced upon exposure of mixed PBMC to HSV, and are profound inducers of NK cell activity (reviewed above), may explain the time course of NK action against HSV-infected target cells.

NK recognition of HSV-infected cells is not unexpected, as HSV down-regulates HLA class I (Hill *et al.*, 1995), and this precise absence is detected by NK cells through loss of inhibitory ligand recognition (Moser *et al.*, 2002). Introduction of HSV-1 ICP47 into target cells can increase their susceptibility to NK cell lysis, as expected from decreased surface HLA class I (Huard and Fruh, 2000). However, it is increasingly recognized that NK cell activation can include ligation of specific activatory receptors in addition to loss of inhibition, and several examples of interactions between virally-encoded proteins and NK receptors have been discovered (Arase *et al.*, 2002; Mandelboim *et al.*, 2001). We believe it is likely that NK cells are involved in controlling virus in recurrent genital HSV-2 lesions that reach clinical threshold, as both NK cell numbers and NK activity were dramatically increased in cells expanded from lesional biopsies compared to normal skin (Koelle *et al.*, 1998b). No activatory interactions between HSV proteins and NK cells are known, but little work was been done in this area. Some early work suggested specific recognition of HSV-1 glycoproteins (Bishop *et al.*, 1984, 1986). In contrast, other work with replication inhibitors and mutant viruses suggest that immediate early gene expression may be sufficient to sensitize HSV-infected targets to NK lysis (Fitzgerald-Bocarsly *et al.*, 1991). With hindsight, it is possible to hypothesize that these HSV glycoproteins were triggering innate immune responses which facilitate NK cell cytotoxicity, or alternatively that direct interactions between NK surface activatory molecules and HSV glycoproteins are occurring . . . we have little real data. NK clones with cytolytic and anti-viral activities against HSV-infected cells from human blood (Yasukawa and Zarling, 1983; Yasukawa and Kobayashi, 1985), expressing clonally distributed NK receptors (Pietra *et al.*, 2000), may assist in determining the molecular basis of NK cell recognition of HSV.

Clinically, low NK cell activity has been linked with severe, usually primary, HSV infections (Biron *et al.*, 1989; Lopez *et al.*, 1983). A homozygous mutation was detected in the Fcγ RIIIa gene of a child with severe HSV infection, and low NK cell number and function, although ADCC function was not abnormal (Jawahar *et al.*, 1996). Tantalizing older literature has linked high HSV disease severity with low PBMC NK activity (Sirianni *et al.*, 1986). As with many immune assays, re-evaluation may be in order using objective measures of HSV severity such as viral shedding rates (Wald *et al.*, 2000), which can differ greatly between immunocompetent persons. Some functional data indicate that NK cells are active in acute HSV lethality models in mice (Rager-Zisman *et al.*, 1987; Bukowski and Welsh, 1986). The difference in lethal dose for 50% of animals (LD_{50}) for acute HSV-1 infection between C57BL/6 and Balb/c mice (Lopez, 1975), discussed above in the context of a TNF-receptor locus, has also been genetically mapped by a second research group to a region containing many NK-cell receptors (Pereira *et al.*, 2001). However, it is not yet known precisely what gene (or genes) is involved.

NKT cells

Several populations of NKT cells, broadly defined as expressing both TCR αβ heterodimers and NK markers such as NK1.1 or CD161, are now recognized (Kronenberg and Gapin, 2002). NKT cells can make large amounts of cytokines such as IL-4 or IFN- γ after activation (Benlagha *et al.*, 2002). In mice, many NKT cells expressing the semi-variant Vα14-Jα281 TCR. Deletion of either CD1d or Jα281 from the germline increases the early pathogenicity of HSV for zosteriform spread in the normally resistant C57BL6 background (Grubor-Bauk *et al.*, 2003). While a subset of NKT cells recognize antigens presented by CD1, the viral or host molecules involved in possible activation of NKT cells by HSV have not been defined. An NKT-like infiltrate in a region of HSV infection has been reported in a human (Taddesse-Heath *et al.*, 2003), but in vitro studies have not documented interactions between murine or human NKT cells and HSV.

TCR$\gamma\delta$ cells

T-cell expressing the gamma and delta T-cell receptor molecules (TCRγδ cells) can recognize HSV-infected cells. Non-MHC-restricted recognition of a peptide from HSV-1 gI has been documented (Sciammas and Bluestone, 1998). TCRγδ cells contribute to HSV-1 clearance in an acute cerebral infection model in mice (Sciammas *et al.*, 1997) and localize to some sites of infection (Liu *et al.*, 1996), but other models have not shown an effect when TCRγδ cells are missing (Nass *et al.*, 2001). Human TCRγδ clones can recognize HSV-1-infected cells in a non HLA-restricted manner (Maccario *et al.*, 1995). These cells can be expanded from the blood by incubation with HSV (Maccario *et al.*, 1993). The jury is still out on the importance of rare

T-cell subsets such as NKT and TCR$\gamma\delta$ cells in human HSV infection.

Additional interactions between HSV and the immune system

Chronic stress has been linked to recurrences of symptomatic HSV disease (Cohen *et al.*, 1999) although not, as yet, to quantitatively measured HSV shedding. Broadly, stressors could affect neuronal events and/or immunologic responsiveness. For example, ciliary neurotrophic growth factor and IL-6 share a receptor subunit, and have been reported to worsen clinical HSV infections in humans or promote reactivation from ganglia in culture (Kriesel *et al.*, 1994, 1997a,b). Manipulations of sympathetic enervation can reduce primary and memory HSV-specific CTL responses in mice and worsen HSV pathogenesis (Leo and Bonneau, 2000). Some data link stress and immune suppression in humans, including relevant effector functions such as NK cell number and activity and lymphocyte proliferation (Zorrilla *et al.*, 2001). Bonneau *et al.* have been studying the relationship between the nervous system, stressors and HSV-specific CD8 CTL activity in C57BL/6 mice. Stress increases the pathogenesis of HSV infection in several models (Bonneau *et al.*, 1991, 1997). Using mice previously immunized with the immunodominant gB1 epitope in a recombinant vaccinia format, the effects of restraint stress and mAb-mediated CD8 depletion were compared for intravaginal or intranasal HSV challenges (Wonnacott and Bonneau, 2002). Both stress and CD8 depletion caused similar, significant increases in acute mortality and increases in vaginal HSV replication.

Very recently, a possible mechanistic link has been suggested by the finding that structured stress decreased the number of HSV-specific CD8 T-cells in the ganglia of HSV-infected mice (Hendricks, 2006). Ongoing work will hopefully elucidate mechanisms that connect stress and the nervous and immune systems.

Summary

The broad host range of HSV and the prevalence of HSV infections in humans have enabled several decades of excellent studies, well beyond the capacity of this review or this reviewer. Despite this, it is fair to say that our understanding of the immune response to HSV is still primitive, and inadequate to address clinical and basic science issues. For example, we do not know if "immunology" can explain the variation in disease severity, how to make an effective vaccine or immunotherapy, or what goes wrong in HSK. HSV research has benefited tremendously from advances in basic immunology, such as the definition of leukocyte subsets, leukocyte-specific proteins, cytokines, and chemokines. The huge effort that has gone into human retrovirus and cancer immunology has led to the development of technique and reagents that have been very useful in the study of acquired responses to HSV in humans. Research accompanying the resurgence of interest in innate immunity over the last decade has revealed that HSV is a strong initiator of innate responses, and that HSV infection can be powerfully modified by innate immunity. Laboratory studies with materials from "outlier" patients with severe HSV, and well-characterized subjects with more routine HSV infections, together with the continuing application of animal models as new key host and viral molecules and pathways are defined, will continue to be rational lines of inquiry. Insights into basic immunology, such as the NIPC and APC identification studies discussed above, are likely to continue to result from HSV research. The challenging synthesis of HSV-specific and broader studies may hopefully lead to clinical advances in the not-too-distant future.

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35

Immunopathological aspects of HSV infection

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Introduction

"What is food to one man is bitter poison to others" Lucretius *De Rerum Natura* (50BCE)

Foreign material entering multicellular organisms triggers a range of defense reactions which, when successful, subjugates and removes the invaders. Invertebrates and plants have natural defense systems, which recognize commonly shared patterns and usually react in a stereotypical manner. Long-lived animals such as vertebrates add to these natural defenses with adaptive systems that show discriminating recognition machinery, complex and varying effector mechanisms and development of persistent or "memory" responses. Under ideal circumstances, immune defense proceeds with minimal or inapparent damage to the host. In other situations, the defense system is less successful and the host tissues become damaged by the reaction. We usually consider the former situation as immunity and the latter as immunopathology. However, in both instances, mechanisms at play may be similar and deciding if the process is one or the other may require Lucretian logic.

With microorganisms, the commonest circumstance that results in immunopathology is where the microbe persists and continues to cause an innate and adaptive response. These, however, prove ineffective to remove or neutralize the agent. Thus the reaction becomes chronic and host tissues become damaged as a consequence. This situation occurs in tuberculosis as well as hepatitis B and C virus infections. Over time, many microbes with a long association with a host species find ways of persisting by evading responses that would either eliminate them or cause too much tissue damage. Human CMV infection in immunocompetent adults provides an example of this scenario (Reddehase, 2002). Other circumstances that result in immunopathology involve settings where one or more components of normal immune defense are compromised for genetic or other reasons. Prolonged severe genital herpes simplex virus (HSV) lesions in AIDS patients with very low CD4+ T-cells represent such an example (Koelle and Corey, 2003). Atopics too often have problems clearing HSV and hence often develop skin and eye lesions (Pepose, 1991a). In addition, some microbes are considered as able to trigger immune reactions that target host components themselves (autoimmune disease) or cause infected cells to undergo neoplastic transformation. The herpesvirus EBV provides an example of the latter in genetically susceptible individuals (Kieff and Rickinson, 2001). There are no undisputed examples wherein herpesviruses cause autoimmune diseases. However, HHV-6 has been proposed to cause multiple sclerosis (Swanborg *et al.*, 2003) and HSV infection may cause an autoimmune corneal inflammatory lesion (Streilein *et al.*, 1997).

Herpes simplex virus is a pathogen that only rarely appears to be involved in immune mediated tissue damage. Characteristically, primary or recurrent infections at superficial mucosal or dermal sites result in viral replication and destruction of most cells that support infection. This process induces an innate inflammatory reaction that contributes to infection control. Some cells, likely Langerhans dendritic cells, leave the site and carry viral antigens to draining lymph nodes where an adaptive response is induced or recalled. After a few days, effectors of adaptive immunity are recruited to the site, initially CD4+ T-cells followed by CD8+ cells, and these T-cells, probably assisted by antibody, complete the task of recovery (Koelle and Corey, 2003). Virus is removed and the inflammatory reaction subsides usually without trace. These events can be judged to represent immunity. When T cell function is impaired, as can happen in AIDS patients, HSV removal is impaired and the inflammatory reaction becomes unusually severe

and prolonged. This situation can be taken to represent immunopathology.

Certain tissue sites are particularly vulnerable to damage by an inflammatory response. These are sites where virus is difficult to dislodge, so the inflammatory reaction becomes prolonged and destructive, or where tissue repair leaves a functionally damaged organ. The eye is the site which best exemplifies such circumstances. In this organ, where inflammation or scar tissue along the visual axis impairs function, HSV infection may permanently impair vision. The most frequent example is herpetic stromal keratitis (SK). This chronic inflammatory reaction damages the stroma and can become sufficiently severe to merit corneal transplantation. About 20% of ocular HSV infections in humans result in stromal keratitis (Pepose *et al.*, 1996). In most instances, these are caused by HSV-1 and result from reactivation from latent infection in the trigeminal ganglion (Pepose *et al.*, 1996). Most of our understanding of the immunopathogenesis of SK comes from studies in animal models, in former times mostly the rabbit and now most usually the mouse.

Human HSV infections may also cause anterior as well as posterior uveitis (retinitis). These are rarer manifestations of HSV infection and much of the damage may result from direct viral damage more than from immunopathology. However, uveitis lesions in the rabbit and mouse models have a definite immunopathological component. Finally, HSV is the cause of a vision destroying reaction in the retina termed acute retinal necrosis (ARN). This lesion occurs both in children infected neonatally or later with HSV-2 as well as in adults where HSV-1 is usually involved (Margolis and Atherton, 1996). Acute retinal necrosis has also been studied in animal models, where lesions were shown to be immunopathological in part (Atherton, 2001).

Inflammatory reactions associated with HSV infection in the peripheral and central nervous systems may also be judged as immune mediated. The best studied example is ganglionitis, an HSV induced lesion that occurs in heterologous hosts but may not be a feature of the natural human disease. Lesions caused by HSV in the CNS are the most dramatic and devastating manifestations of HSV infection of humans. This rare disease is usually caused by HSV-1 infection in adults and is mostly a direct virologic lesion. However, immunopathological events such as demyelination may also occur in a few cases. In infants and children, encephalitis is more commonly associated with neonatal infection with HSV-2 and this lesion appears to be the direct result of a lytic virus infection.

Finally, there are some chronic inflammatory reactions that have been associated with HSV infection especially with the widespread use of modern technology to detect viral DNA. Some reports suggest that the virus or an immune response against it accounts for such HSV associated diseases as erythema multiforme, arteritis, Alzheimer's disease, Bell's palsy and Behcet's disease.

Herpes infections and ocular disease

At least four human herpesviruses have been implicated as causes of ocular disease. Two alphaherpesviruses, HSV and varicella zoster virus (VZV), the betaherpes virus CMV and the gammaherpesvirus EBV. CMV is a cause of retinitis, a lesion found only in immunosuppressed individuals, the majority of which were formerly AIDS patients, but now transplant recipients, especially recipients of bone marrow (Holland *et al.*, 1996). With the widespread use of protease inhibitors to control HIV, CMV retinitis is now mainly a disease of transplant recipients. The lesion itself is likely a direct consequence of viral replication in retinal cells. EBV is an occasional cause of SK lesions. These are characterized by an abundance of lymphoma like cells, in the stroma that are presumed to be mainly B cells (Matoba, 1990).

More commonly both HSV and VZV cause lesions in the anterior segment, principally the cornea. Both HSV and VZV can also cause uveitis and acute retinal necrosis (ARN).

Keratitis in humans

Both HSV and VZV can infect multiple structures in the eye. Lesions caused by HSV are much more common. The incidence of HSV ocular disease ranges from 4.1–20.7 cases/100 000 patient years representing the commonest single infectious cause of vision impairment in the western world (Pepose *et al.*, 1996). Of the three general types of HSV corneal disease, Infectious Epithelial Keratitis (IEK) is the most common lesion and appears to be a result of the direct effect of viral infection. Both disciform keratitis (HSV endotheliitis) and SK are thought to be mainly the consequence of immune mediated mechanisms rather than direct viral damage. IEK lesions are a result of viral replication and spread in the superficial epithelial layer of the cornea. This condition is usually self-limiting and no permanent corneal damage results. The quick remission seen with timely antiviral therapy suggests a simple viral cytolytic mechanism. However, virus invariably infects nervous ramifications in the cornea that have free ends within the epithelial layer, thus allowing retrograde transport and establishment of latency (Shimeld *et al.*, 2001). In addition, as a consequence of epithelial damage, virus can spread to the underlying stromal keratocytes and cause what is usually termed a necrotizing

form of stromal keratitis (Liesegang, 1999). This terminology is not used by many ophthalmologists since necrosis also occurs in immune mediated SK (T. P. Margolis, personal communication, 2003).

Disciform keratitis (DK) is a lesion in which the corneal endothelium is the primary site of damage. This form of ocular disease appears immunopathological based upon the fact that early intervention with corticosteroids leads to complete resolution (Liesegang, 1999). In DK, the inflammatory reaction of the endothelium sometimes results in secondary stromal and epithelial edema but there is usually no stromal infiltrate or neovascularization. One of the characteristic findings is the demonstration of keratic precipitates or KP (Liesegang, 1999). The exact nature of the KP is unknown but they could be aggregates of macrophages or NK cells attracted by the immunoglobulins on the surfaces of infected cells (Liesegang, 1999). An alternative idea is that KP represent cytotoxic T-cells recognizing viral epitopes on the endothelial cells (Liesegang, 1999). The role of live virus in disease development is supported by finding antigens, live virus and DNA in the anterior chamber and perhaps also corneal endothelial cells (Kaufman *et al.*, 1971; Sundmacher and Neumann-Haefelin, 1979a). It has been postulated that productive infection of the endothelial cell elicits a cellular and humoral immune response (Sundmacher and Neumann-Haefelin, 1979b), but this evidence is only circumstantial. Alternative suggestions include a possible delayed type hypersensitivity reaction to persisting HSV antigens within the stroma or the endothelium (Pepose, 1991b). It is difficult to resolve the nature of DK pathogenesis since animal models to study it are less than ideal. Disciform disease is seen in rabbits with an intracorneal injection of soluble viral antigen (Williams *et al.*, 1965). Using the rabbit model for DK, some have suggested that the lesions involve immune complex formation and antibody dependent cell mediated cytotoxicity (Meyers and Chitjian, 1976).

Inflammation of the corneal stroma (SK) as a result of HSV-1 (rarely HSV-2) infection can lead to a blinding immuno-inflammatory lesion of the stroma. This only accounts for approximately 2% of initial episodes of ocular disease but approximately 50% of recurrent ocular HSV disease (Norn, 1970). A similar, but even more devastating lesion can be caused by VZV infection. Fortunately, this is quite rare and also usually occurs as a consequence of reactivation (zoster). The infections usually heal quickly unless the patient is immuno-suppressed (Pepose *et al.*, 2003). Recurrent lesions can be very severe and most difficult to treat and control. Frequently, corneal lesions are accompanied by conjunctivitis, anterior uveitis and lipid keratopathy (Pepose *et al.*, 2003). If the virus is not controlled, it spreads to involve the iris and the corneal stroma. Stromal lesions can become sclerotic and very persistent and is believed to be immune mediated, however, the mechanism is not known and is difficult to study. Patients often lose sensitivity of the cornea and involuntary physical damage can result in secondary bacterial infection.

Table 35.1. Immunopathological basis for SK in humans

Lesions in the absence of virus or viral antigens
Indefinite corticosteroid therapy
Uncommon in immunosuppressed individuals
T-lymphocytes with reactivity to viral epitopes detectable in corneas with chronic lesions

Several observations suggest the operation of an immune etiology behind HSV induced SK (see Table 35.1). These include the fact that the lesions are persistent and are manifest well beyond the time that virus or viral antigens can be demonstrated. Lesions often need to be managed with indefinite corticosteroid treatment and reactivation lesions, except initially, do not benefit from acyclovir antiviral treatment. Also making a case for the pathogenesis of SK involving immunopathology is the fact that the lesion is very uncommon in immunosuppressed patients. Finally, clones of T-lymphocytes reactive to viral epitopes and possessing cytotoxic activity can be cultured from corneas showing chronic SK lesions (Verjans *et al.*, 1998; Koelle *et al.*, 2000).

Approximately 90% of patients maintain good visual acuity despite prolonged disease. However, in many cases resolution of inflammation is associated with a permanent loss of vision resulting from corneal scarring and ulceration. This necessitates treatment by corneal transplantation, which in itself can sometimes be a high risk factor for recrudescent herpetic keratitis (also called newly acquired herpetic keratitis) (Remeijer *et al.*, 1997) and super-infection with a different strain (Remeijer *et al.*, 2002)

The corneal stroma may be affected by several mechanisms; this may be secondary to disease of the epithelium (IEK) or endothelium (DK) or as a stromal edema resulting from a damaged endothelium. In humans, SK manifests itself in two primary forms that are perhaps mis-termed necrotizing SK and immune SK (Liesegang, 1999). While the former is thought to result from direct viral invasion of the stroma, chronic immune mechanisms, possibly of an autoimmune nature (yet unproven), are suspected in pathogenesis of the latter (Pepose *et al.*, 1996). These divisions are not mutually exclusive and necrosis can definitely occur in the immune form of disease. Intact virions and antigens can be detected in corneal keratocytes, endothelial cells and foci of epithelial cells

in specimens from patients with acute (necrotizing) stromal keratitis (Kobayashi *et al.*, 1972; Metcalf and Kaufman, 1976). This suggests that replicating virus and the resulting host inflammatory response leads to stromal cell destruction. This acute necrotizing form of SK eventually may become chronic, then considered to be the immune form of SK, when viral antigens are no longer present. The signs of SK are generally quite variable but they include the influx of a large number of different kinds of cells including polymorphonuclear leukocytes (PMN), macrophages, Langerhans' cells, natural killer (NK) cells, plasma cells and T-lymphocytes (Pepose *et al.*, 1985a; Youinou *et al.*, 1985, 1986; Miller *et al.*, 1993). In chronic herpetic SK in humans, the predominant population are macrophages and T-lymphocytes (Youinou *et al.*, 1985). Excess neovascularization also occurs in some patients.

The original mechanism proposed for the pathogenesis of the immune form of SK focused on the role of anti-HSV antibodies. This was based on the finding that rings (Wessely rings) seen in the mid-stroma of the cornea in immune stromal keratitis were positive for IgM, IgG and IgA (Meyers-Elliot *et al.*, 1980). Herpes virus particles have been demonstrated in these rings, many of them defective or incomplete (Meyers-Elliot *et al.*, 1980). In addition, viral antigens have been found localized in the keratocytes of the corneal stroma in transplanted corneas (Youinou *et al.*, 1986; Easty *et al.*, 1987). Hence it has been speculated that viral antigens trapped in the stroma acted as a nidus for deposition of anti-HSV antibodies that fix complement and leads to cellular damage (Pepose *et al.*, 1996). Viral antigens can also be presented to the infiltrating T-lymphocytes. In clinical specimens, increased levels of class I and II HLA antigens have been noted in areas of the greatest infiltrate, suggesting active presentation of antigens (Pepose *et al.*, 1985b). Both CD4+ and CD8+ T-cells occur in chronic herpetic SK with the former dominating the total T-cell numbers (Youinou *et al.*, 1986). Most of these cells are reactive against HSV antigens with the CD4+ subset reactive to peptide epitopes from UL21 and UL49 tegument proteins of HSV (Verjans *et al.*, 1998; Koelle *et al.*, 2000). They do not apparently recognize antigens derived from corneal tissues which would provide evidence for an auto-immune mechanism (Verjans *et al.*, 1998). Corneal derived CD4+ cells have been shown to possess cytotoxic activity suggesting the possible operation of this mechanism in stromal cell injury (Verjans *et al.*, 1998; Koelle *et al.*, 2000).

Animal models for SK

Understanding the pathogenesis of human SK from clinical observations, transplant material and the occasional samples obtained at biopsy is difficult. Fortunately, convenient animal models exist wherein HSV infection of the eye reproducibly generates a stromal inflammatory response. Moreover, this appears to reflect human immune SK at least before it is treated. The usual animal models are the mouse and rabbit with the latter now rarely used except for studies on therapy. Events in SK pathogenesis are mainly studied in primary infection of various mouse strains. Since human SK is most commonly a sequel to reactivated HSV, a better animal model should be one where lesions follow reactivation. Such models have in fact, been described for both mice and rabbit (Myers-Elliot and Chitjian, 1983; Shimeld *et al.*, 1989) but these are expensive and inconvenient and have contributed minimally to the understanding of pathogenesis. Rabbit reactivation can be achieved by ocular iontophoresis but this seldom gives rise to SK lesions (Myers-Elliot and Chitjian, 1983). The mouse reactivation model can be achieved by infecting mice under a cover of neutralizing antibody, then after some weeks asymptomatic animals are exposed to UV light. In usually a minority of animals, virus reactivates and generates an inflammatory reaction in the stroma (Shimeld *et al.*, 1989). Few papers have employed the model, and the results of these usually support the basic findings of the primary infection model; namely that SK is an immuno-inflammatory lesion mainly orchestrated by CD4+ T cells (Shimeld *et al.*, 1996).

The primary infection model usually uses strain HSV–1 RE and involves virus application to a lightly scratched cornea. Replication begins in epithelial cells of the cornea and usually the conjunctiva, but in immuno-competent mice rarely spreads to involve stromal cells or cells in uveal tissue. Characteristically, the viral replication events are over by 5–6 days and viral gene expression, as judged by protein detection or viral mRNA, are undetectable beyond a further 2–3 days (Babu *et al.*, 1996). Viral DNA, however, can be detected for prolonged periods although copy numbers, detectable by real-time PCR, do not exceed 2000–5000 copies per cornea by 14 days p.i. (our unpublished results). When looked at with an ophthalmoscope, the initial viral replication events are accompanied by a barely detectable inflammatory reaction with new blood vessel growth from the limbus (the location of blood vessels at the edge of the vessel free normal cornea) the most obvious feature. This is often referred to as the preclinical phase, although in fact with appropriate tests is readily observable.

Innate reactions to infection in the mouse model

HSV infection of the corneal epithelium sets off a range of humoral and cellular events that taken together help contain infection. Unfortunately, some of these also set the stage for subsequent immunopathology. A prominent early cellular event is the influx of polymorphonuclear neutrophils (PMN). This occurs mainly into stromal tissues subjacent to the infected epithelium. Such PMN escape

from blood vessels at the limbus presumably in response to signaling molecules generated from virus infected cells. The nature of such signaling molecules is unclear but several chemokines, including those known to be chemotactic to PMN, can be demonstrated within 12 hr p.i. (Su *et al.*, 1996; Thomas *et al.*, 1998)

The PMN response is at its peak around 48 hrs and it seems that this response helps control viral replication. Thus depleting PMN with specific monoclonal antibodies results in more intense and prolonged virus infection in the cornea (Tumpey *et al.*, 1996; Thomas *et al.*, 1997). Moreover, PMN suppressed animals may succumb to encephalitis since virus now spreads to the brain. Such observations indicate that PMN are part of the antiviral defense system although it is unclear how this function is performed. Accordingly, virus infected cells and PMN are usually not in direct contact implying that the protective function is indirect. Ideas for the mediation of such defenses include IFNγ and TNFα production as well as nitric oxide production by PMN (Daheshia *et al.*, 1998a). This topic has not been fully explored using, for example, knockout mice and other means of implicating potential antiviral mechanisms.

The PMN response to virus is not only a defense reaction. Indeed products released from PMN have been proposed to contribute to corneal damage possibly unmasking autoantigens subsequently involved in the immunopathology (Thomas *et al.*, 1997). In addition, PMN contribute to the process of neovascularization, a prominent feature of SK and a necessary step in its pathogenesis (Zheng *et al.*, 2001a; Lee *et al.*, 2002a). It appears that PMN may be a source of angiogenesis factors such as VEGF as well as tissue degrading enzymes which breakdown the stromal matrix and facilitates the growth of new blood vessels. One such enzyme released by the granules of activated PMN is MMP9 (Lee *et al.*, 2002a). Since infected mice given the MMP-9 inhibitor TIMP-1 as well as MMP9-/- mice have reduced angiogenic and SK responses, MMP9 appears to be intricately involved in SK pathogenesis (Lee *et al.*, 2002a).

Although PMN dominate the early inflammatory reaction to ocular HSV infection, other cell types can also be demonstrated. These include macrophages, dendritic cells (DC), NK cells but not B or αβ TCR T cells. The roles for these other cell types have received minimal investigation. It is likely, however that the macrophage is a source of angiogenic factors such as VEGF and FGF as well as the angiogenic CXC chemokines. For example MIP-2 (CXCL8) appears important since infected mice lacking the receptor for MIP-2 have an impaired PMN response (Banerjee *et al.*, 2004a). In addition, in vivo neutralization of MIP-2 in HSV infected mice reduces PMN migration (Yan *et al.*, 1998). Macrophages, along with DC, also act as a source of cytokines demonstrable early after infection. Most prominent of these are IL-1 and IL-6, both of which can also be produced by virus infected epithelial cells themselves (Tran *et al.*, 1998; Kanangat *et al.*, 1996). Indeed, it could be that these two cytokines are critical signaling molecules responsible for the many paracrine events set off by virus infected epithelial cells. The other early events described include the production of IL-12, VEGF and TNFα, but none of these are thought to be products of virus-infected cells themselves (Zheng *et al.*, 2001b; Kumaraguru and Rouse, 2002). We have demonstrated that IL-6, for example, can cause macrophages *in vitro* to generate VEGF (Banerjee *et al.*, 2004a), and IL-1 is well known to cause mononuclear cells to produce TNFα and other cytokines (Neta *et al.*, 1992). Our recent findings also indicate that within HSV infected corneas, IL-1 maybe responsible for IL-6 expression, which in turn upregulates VEGF production (Biswas *et al.*, 2004).

The cytokine IL-12 appears as a pivotal molecule in SK pathogenesis. Knockout mice, for example, unable to produce IL-12 have only mild SK lesions (Osorio *et al.*, 2002). The source of IL-12 following HSV infection remains to be clarified, since as mentioned it does not appear to be HSV-infected cells themselves (Kumaraguru and Rouse, 2002) However conceivably viral DNA that has pathogen associated molecular pattern (PAMP) activity could represent such a stimulus (Zheng *et al.*, 2002). The most likely producer cell types are DC and macrophages. The DC initially involved would seem to be the resident cells only recently demonstrated as present in normal non-inflamed corneas (Hamrah *et al.*, 2003). A prominent feature of the injured cornea, including that caused by HSV, is the invasion of Langerhans DC, likely from the conjunctiva, into the cornea (Jager *et al.*, 1991). However, this event takes several days to occur. Likely such cells also act as a source of cytokines and chemokines but their major function in SK pathogenesis is transport of viral antigens to lymphoid tissue where the adaptive immune response is initiated (discussed later).

The cytokine IL-12 has several downstream effects that impact on SK pathogenesis. The primary effect is induction of IFNγ production by cells with IL-12 receptors. Although not proven in the eye, the most likely cells that respond and produce IFNγ are natural killer (NK) cells. Such cells in non-ocular systems have been shown to be important for resistance to HSV. In fact, removing them results in heightened susceptibility (Rager-Zisman *et al.*, 1987). An early study of SK indicated that NK removal ameliorated SK (Tamesis *et al.*, 1994; Bouley *et al.*, 1996), although this issue warrants further investigation. Whatever the source of IFNγ, this molecule appears to be intricately involved in antigen processing as well as other events critical for

SK pathogenesis. These include up-regulation of the cell adhesion molecule PECAM-1 on vascular endothelial cells, at the limbus (Tang and Hendricks, 1996). This is a necessary step for normal PMN invasion as evidenced by the fact that neutralization of IFNγ or PECAM-1 results in diminished PMN ingress (Tang and Hendricks, 1996).

The importance of IFNγ in facilitating cell migration is further underscored by studies with human corneas. Stimulation of human corneal cells in vitro with IFNγ, and also IL-1 and TNFα, rapidly up-regulates ICAM-1 expression (another cell adhesion molecule that participates in the adhesion and extravasation of cells) (Pavilack *et al.*, 1992). IFNγ also upregulates MHC Class II expression on the antigen presenting cells involved in the induction of the initial antigen specific CD4+ T-cell response in local draining lymph nodes (Dreizen *et al.*, 1988; Foets *et al.*, 1991). On the other hand, IFNγ could help modulate lesion development since it also induces angiostatic chemokines such as IP-10 and MIG (Lee *et al.*, 2002b). Accordingly the IL-12 response to HSV infection indirectly impacts on both inflammatory and regulatory effects on SK.

In Fig. 35.1 several critical events are shown that are set into play by HSV during the first 6–7 days postinfection. By the end of this often-called preclinical phase, the corneal tissues show little or no damage. The epithelium is fully intact, the stroma has few if any inflammatory cells and cytokine/chemokine levels have fallen significantly. The most obvious sign of change is a neovascular bed that continues to expand slowly beyond the limbal region. Nevertheless, in spite of the quiet appearance, notable changes begin to occur which constitute the true immunopathological events of SK. Accordingly, the T-cell orchestrators begin to invade via the new blood vessels and an intense inflammatory response ensues. This becomes obvious upon ophthalmoscopic examination and is frequently referred to as the clinical phase.

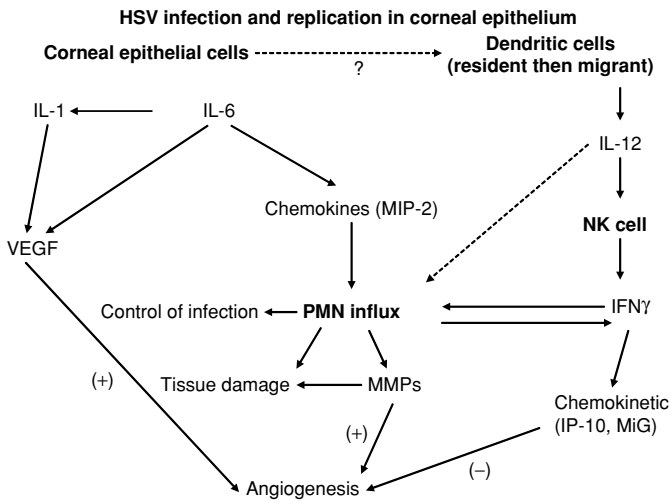

Fig. 35.1. Some early critical events occurring after HSV-1ocular infection.

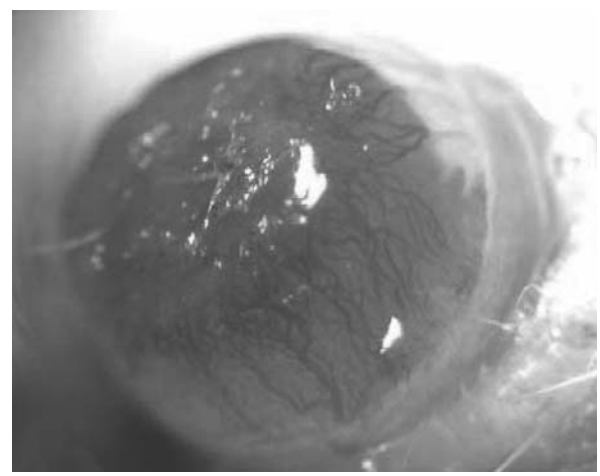

Fig. 35.2. Corneal blood vessels at the peak of HSK lesions in the mouse (day 15) with evidence of corneal opacity, necrosis and epithelial ulcers.

Adaptive immunity in SK

Migration of T-cells that express appropriate homing molecules escaping from the newly established blood vessels represents a crucial step in SK pathogenesis. Mice without T-cells never develop typical SK lesions (Metcalf *et al.*, 1979; Mercadal *et al.*, 1993), but do if given T-cell transfers (Russell *et al.*, 1984; Mercadal *et al.*, 1993). Although debated early on, most investigators now agree that CD4+ T-cells, with the type 1 producing cytokine phenotype, are the main aggressors in HSK (Niemialtowski and Rouse, 1992; Mercadal *et al.*, 1993). Such cells trigger the invasion of non-specific inflammatory cells, surprisingly once again dominated by PMN, giving rise to a peak response around 15 days after initial infection. The inflammatory response considerably thickens the stroma and neovascularization continues almost reaching the central cornea (see Fig. 35.2). Severe lesions have areas of necrosis and epithelial ulcers and uveal tissues may also be involved. The lesional T-cells, which account for only a minority of the inflammatory cells present, are mainly CD4+ T-cells. Judging from a variety of approaches, the principal cytokine necessary for the lesion expression is IFNγ (Tang and Hendricks, 1996; Deshpande *et al.*, 2002). However, SK can still be induced in animals lacking this cytokine (Bouley *et al.*, 1995). In cases where lesions do diminish in severity, the cytokine IL-10 is upregulated (Babu *et al.*, 1995). Furthermore, the artificial expression of IL-10 or IL-4 early in the syndrome can markedly diminish lesions (Daheshia *et al.*, 1998b).

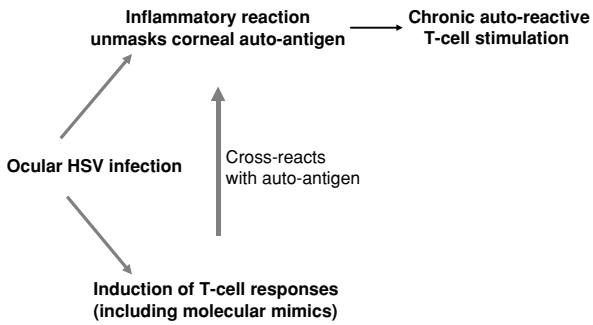

Fig. 35.3. The autoimmunity model of SK pathogenesis.

Such observations indicate that CD4+ Th1 are the principal aggressors but if a type 2 response can be induced, lesions will resolve. Whether such ideas can be applied usefully to the human system warrants investigation.

Very recently the severity of SK lesions was shown to be modulated by a second species of CD4+ T-cells (Suvas *et al.*, 2004). These were CD4+CD25+ T-regulatory cells (T_{reg}) found operative in autoimmune inflammatory lesions (Shevach, 2000). Accordingly, in animals unable to generate T_{reg} responses, SK lesions were more severe and animals more susceptible to a low dose of infection (Suvas *et al.*, 2004). In addition, there is evidence that the CD8 T cell response to HSV provides a protective function against SK (Mercadal *et al.*, 1993; Gangappa *et al.*, 1999; Banerjee *et al.*, 2004b, 2005). The mechanisms by which T_{reg} or CD8+ T-cells exert controlling effects on SK expression are not currently understood but are being actively explored.

A central issue in SK pathogenesis is the nature of antigens recognized by the CD4+ T-cell orchestrators and if such recognition occurs in the extra-lymphoid or lymphoid sites (or both). This issue becomes of interest since at the time when T-cells invade the cornea, replicating virus has usually disappeared (Babu *et al.*, 1996). Moreover, certainly at the time of peak lesions (15 days), the presence of viral antigens in stromal tissues cannot be demonstrated (Babu *et al.*, 1996). Conceivably, viral peptides expressed by DC could still be present in the cornea and draining lymph nodes, although usually T-cell target peptides turn over within 2–3 days after protein processing. Since new protein formation appears to have ceased by 6 days p.i., it is difficult to support the logical notion that peptides derived from viral proteins are the target antigens recognized by the $T_{aggressors}$.

An alternative concept is that viral specific T-cells are initially responsible for the immunopathology but subsequently the chronic phase is maintained by an autoreactive response (Deshpande *et al.*, 2002). Here the idea is that the virus infection results in unmasking of the some corneal autoantigen (see Fig. 35.3), tolerance is broken and the autoreactive T-cells induced are responsible for orchestrating lesions. A modification of this idea favored by the Cantor group is that the autoimmune process is set off by some viral peptide sharing reactivity to the unmasked corneal autoantigen (Zhao *et al.*, 1998). Thus the initial antiviral response subsequently becomes sustained by autoreactive $T_{aggressor}$ cells. This concept of molecular mimicry has aroused much interest and discussion (Deshpande *et al.*, 2002). Its best support comes from studies on closely related inbred mice. Here it would seem that the UL6 protein of HSV possesses molecular mimicry with an autopeptide that in fact represents a sequence also found on an immunoglobulin isotype (Zhao *et al.*, 1998). The molecular mimicry idea is not accepted by other groups for a number of reasons (See Table 35.2). Most, especially the UL6 proteins of HSV appear not to induce T cell responses in animals following infection with HSV (Deshpande *et al.*, 2001a). In humans also the UL6 protein appears not to be recognized (Verjans *et al.*, 1998; Koelle *et al.*, 2000; Ellison *et al.*, 2003).

Table 35.2. Lack of evidence for molecular mimicry between HSV UL6 peptide and corneal antigen

1. Failure to demonstrate an immune response directed to UL6 peptide after HSV ocular infection	Deshpande *et al.*, 2001a
2. Ocular infection with vaccinia virus expressing UL6 fails to induce HSK	Deshpande *et al.*, 2001a
3. CD4+ T-cells that are apparently tolerized to molecular mimics are able to induce SK lesions upon adoptive transfer to SCID mice	Thomas and Rouse, 1998
4. T-cells extracted from human corneas show no reactivity to HSV UL6	Verjans *et al.*, 1998; Koelle *et al.*, 2000
5. T-cells extracted from human corneas are not reactive to corneal antigens	Verjans *et al.*, 1998
6. Variation in clinical presentation of SK is not due to genetic variation in the UL6 epitope	Ellison *et al.*, 2003

An alternative idea to explain how CD4+ T-cells become activated is that the inflammatory process could be initiated by viral antigen recognizing T-cells, but subsequently is maintained by cells of the effector memory phenotype that escape into the cornea because of the highly permeable neovascular bed. Such cells, in turn, become activated by inflammatory molecules initially released by viral antigen reactive cells. The responding cells, release inflammatory

cytokines and so the process continues (see Fig. 35.4). This idea is supported by the observation that abundant non-antigen T-cells can be demonstrated and that it is possible to develop lesions identical to SK in animals whose T-cells are genetically incapable of recognizing viral antigens (Gangappa *et al.*, 1998; Deshpande *et al.*, 2001b; Banerjee *et al.*, 2002). Such was shown in several T-cell transgenic mice on SCID or RAG–/– backgrounds whose recognition repertoire did not include HSV antigen recognition (Gangappa *et al.*, 1998; Deshpande *et al.*, 2001b; Banerjee *et al.*, 2002). In these models, the chronic source of activating cytokines were cells dying of HSV infection since in this instance virus persisted and spread to the stromal site of inflammation (Gangappa *et al.*, 1998; Deshpande *et al.*, 2001b; Banerjee *et al.*, 2002).

Other ideas have also been advocated to explain which agonists drive SK, especially in the chronic phase, but the issue remains unresolved. The candidate agonists include PAMP expressed by virus, superantigen expression and inflammatory reactions driven by stress proteins (Deshpande *et al.*, 2002). Currently, a favored idea is that the HSV DNA could itself be pro-inflammatory because of its high content of bioactive CpG containing deoxynucleotide motifs (Zheng *et al.*, 2002). Such ideas await verification.

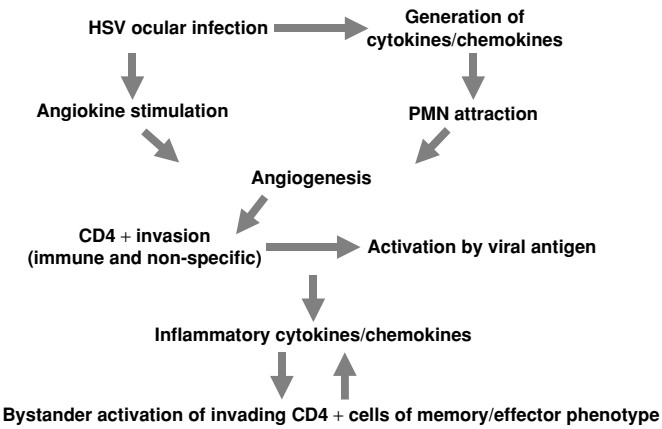

Fig. 35.4. Bystander activation model for SK pathogenesis.

Human Uveitis

Herpes simplex virus infection of the iris, ciliary body and the choroid results in an inflammatory condition known as HSV uveitis. This lesion usually accompanies SK or DK but occasionally it can stand alone. In many cases, it can occur in the absence of a previous history of HSV infection. Ophthalmoscopic examination reveals the presence of fine keratic precipitates (KP) and anterior chamber inflammation that ranges from mild to severe (Liesegang, 1999). The virus can be detected in the inflamed iris by electron microscopy. There seems to be a lack of consensus about the isolation of infectious virus from the anterior chamber of patients with HSV uveitis (Kimura, 1962; Sundmacher and Neumann-Haefelin, 1979b). This disparity may, however, result from differences among patients in the relative concentrations of anti-HSV antibodies to the virus in the anterior chamber that is needed to clear virus. Interestingly, intra-ocular antibodies recognize different viral antigens than those recognized by antibodies in the systemic immune response (Peek *et al.*, 2002). The implications of this finding are unclear but possibly persisting intraocular antibodies could be involved in the development of secondary uveitis by the formation of toxic immune complexes. Currently, the only evidence supporting an immunopathological basis for HSV uveitis in humans is the benefit of treatment achieved with administration of topical corticosteroids along with systemic acyclovir (Liesegang, 1999). Anterior chamber uveitis can precede ARN, the latter lesion initially virologic, but later on includes immune mediated events (discussed later).

The rabbit model for HSV uveitis

The possible immunopathological nature of HSV uveitis has been revealed in studies with the rabbit model (Oh, 1976). Injection of live virus directly into the vitreous humor (intravitreal injections) of the rabbit eye results in a slowly progressing inflammation of the uveal tract. Eventually an HSV neutralizing antibody response in the infected eyes, clears the virus. Only live virus is capable of producing primary uveitis. However in eyes that have recovered, secondary uveitis can be induced by injection of inactivated virus (Oh, 1976). The disease kinetics of this secondary disease is faster than that seen with the primary infection. Such results indicate that in cases of secondary uveitis the pathogenic mechanism is likely to be immune mediated.

Acute retinal necrosis (ARN) in humans

This dramatic and devastating lesion can be caused by VZV or more commonly by HSV. Lesions can result from primary or recurrent infection and in the case of VZV can stand alone or accompany chicken pox or zoster (Ganatra *et al.*, 2000). Without treatment the virus destroys the retina in 7–14 days. The initial stages of acute retinal necrosis (ARN) appear to be the direct result of viral damage but later stages involve immune mediated events.

Either HSV-1 or 2 can be involved. HSV-2 is usually the cause in infants, children and young adults (average age 27). In adults HSV-1 is usually the culprit (average age 50). With

HSV-2 induced ARN there is often a history of encephalitis and meningitis. It is thought that virus remains in the brain after such lesions, but then passes from that site to affect the retina (Margolis and Atherton, 1996). Typically, ARN starts off as a virologic lesion, but subsequently becomes immune mediated. The vitreous fills up with inflammatory cells and occlusive vasculitis can occur which deprives the retina of its blood supply (Margolis and Atherton, 1996). In addition, both CD4+ and CD8+ T-cells, possessing cytolytic and cytokine secreting functions, have been isolated from intraocular fluids of patients. These cells have been found to be reactive with HSV UL46 and UL47 encoded tegument proteins VP11/12 and VP13/14 (Verjans *et al.*, 2000).

In adults where HSV-1 is the usual cause, ARN is most commonly unilateral and usually associated with encephalitis. However there are two cases of retinitis that have occurred years after recovery from encephalitis (Margolis and Atherton, 1996).

The mouse model of ARN

Both the mouse and the rabbit model have been employed to study the pathogenesis of ARN and have yielded interesting clues for pathogenesis. If injected intra-cerebrally, virus spreads to the retina via the optic nerve (Atherton, 2001), providing evidence that a similar effect could occur in humans accounting for ARN long after an episode of encephalitis (Margolis and Atherton, 1996). ARN can also be induced by injecting HSV into the anterior chamber. Such infection usually fails to cause lesions in the injected eye, that is presumed to be protected by an IFNα response (Atherton, 2001). However, after a few days the contralateral eye develops a severe retinitis. This response is assumed to be immune mediated since it is of much milder extent in T-deficient mice. Moreover, reconstituting such mice with CD4+ immune T-cells, but not CD8+, restores lesion expression (Atherton, 2001).

Taken together, results from the mouse and human studies support the idea that a combination of viral infection of the retina and virus specific T-lymphocytes is likely involved in the pathogenesis of ARN.

Herpes simplex virus in the nervous system

In humans the most dramatic and devastating disease associated with HSV infection is herpes simplex encephalitis (HSE). Fortunately this is a rare syndrome for it is usually lethal or leaves patients with serious neurological damage. In adults, HSE is usually caused by HSV-1 and can occur following primary or more commonly recurrent infection (Whitley, 2001). Lesions are mainly considered to be the direct cytolytic effect of the virus. However, inflammatory reactions occur that include both CD4+ and CD8+ T-cells. In about 3% of adult cases demyelination has been noted likely a consequence of a T cell mediated immunopathological reaction (R. J. Whitley, 2003, personal communication). HSE in infants is occasionally associated with involvement of the retina (ARN).

Whereas HSE is a very rare disease in humans, heterologous hosts infected with this alphaherpesvirus are far more likely to suffer from HSE. Thus primary infection of susceptible mouse strains, especially with HSV-2, results in spread to the CNS and death from encephalitis (Hudson *et al.*, 1991; Whitley, 2001). Whereas in humans there is no evidence of neurotropic strains of HSV, in mice some viral strains are far more neurotropic than others (LaVail *et al.*, 1997). In some cases, the neurotropism has been associated with known amino acids in a single protein (Diefenbach *et al.*, 2002). The rodent form of HSE largely represents a direct effect of viral destruction, but immunopathology can play a role (Kastrukoff *et al.*, 1993; Hudson and Strelein, 1994). Multifocal brain demyelination (MBD) has been reported in susceptible mouse strains upon lip inoculation with HSV-1 and immunosuppression prevents the development of such lesions (Kastrukoff *et al.*, 1987; Kastrukoff *et al.*, 1993). Of the two major T-subsets, CD8+ T-cells appear to be involved in the focal lesions of the brain and depleting such cells prevents lesion development (Hudson and Streilein, 1994). Other studies indicate that CD8+ T-cells play both a protective and pathogenic role in encephalitis (Anglen *et al.*, 2003). These studies evaluated the role of such cells in stress induced HSE. If present prior to an infection, protection ensues, possibly by limiting the HSV replication and spread within the CNS; the delayed entrance of CD8+ T-cells could result in pathology, based on limited evidence (Anglen *et al.*, 2003).

Ganglionitis

A characteristic feature of all alphaherpesviruses is that they succeed in gaining access to sensory nerve fibers during primary infection and pass by retrograde axonal transport to the nerve cell bodies in the appropriate ganglion. At that site, whereas some neurons appear to support a productive infection, in others an alternative replication cycle is initiated that results in latency (Roizman and Knipe, 2001). With HSV, at least, latency is thought to be an immunologically cryptic situation, since the viral transcripts expressed have no protein product (Roizman and Knipe, 2001). Latency in a particular neuron can be maintained indefinitely, but some infected neurons restart the productive cycle and progeny virus spreads by anterograde transport to peripheral sites, such as the cornea. In their homologous hosts, alphaherpesviruses rarely spread

to the CNS after primary infection. Such events are quite common in heterologous hosts such as HSV in the mouse. Moreover, reactivation in homologous hosts often results in recrudescent lesions, but such are rare in heterologous situations. In the mouse latently infected with HSV, occasional neurons undergo reactivation (about 1 neuron in 5 days) and this induces a notable local inflammatory reaction (Feldman *et al.*, 2002). This likely prevents widespread dissemination in the ganglion.

Another event that characterizes HSV infection in heterologous hosts is a marked and prolonged ganglionitis that occurs after primary infection (Shimeld *et al.*, 1995; Liu *et al.*, 1996). This represents an immune mediated event that mainly involves CD8 + T-cells (Liu *et al.*, 1996; Liu *et al.*, 2000; Khanna *et al.*, 2003). The CD8 + T-cells seemingly function to purge productively infected neurons of virus, rather than killing them by a cytotoxic mechanism (Liu *et al.*, 2000; Khanna *et al.*, 2003). Hence there is no tissue damage and strictly speaking no pathology. Currently, the relevance of heterologous ganglionitis is not understood nor is it known if a similar phenomenon occurs in infected human ganglia. Ganglionitis, may be another example of events that occur only in heterologous hosts infected with alphaherpesviruses.

The course of events in the mouse ganglion has been carefully studied and they tell an intriguing story (Liu *et al.*, 1996; Liu *et al.*, 2000; Khanna *et al.*, 2003). The initial events involve viral replication and an inflammatory cascade that resembles that described for SK. However, after 7–10 days, CD4+ cells appear to enter and orchestrate subsequent events in SK whereas a remarkably high percentage of the inflammatory cells in the ganglia are CD8+ T-cells. Moreover, most of these are viral antigen specific and maintain this phenotype for months, which would indicate their continuous activation by antigen. However, demonstrating such antigen has proven impossible and most would agree that, after 10 days or so, virus is latent in neurons. The ganglionitis studies, however, imply that some antigen might be expressed by neurons but these are not sacrificed (Liu *et al.*, 2000; Khanna *et al.*, 2003); instead they are spared by the ability of the CD8+ T-cells to purge them of their offending virus (Liu *et al.*, 2000; Khanna *et al.*, 2003). These ideas remain to be proven and shown to be not purely a murine idiosyncrasy.

Other possible HSV induced immune mediated conditions

Herpes simplex virus affects the majority of mankind and it usually persists in some form in all it infects. Indeed, the latest sensitive molecular approaches have revealed that HSV DNA can be found in many tissues previously not recognized as an infection site. Such observations have led to the speculation that HSV could contribute to the cause of several chronic inflammatory diseases. For example, some have associated HSV with Alzheimer's disease based on the high correlation between HSV-1 in the brain and Alzheimer's disease (Pyles, 2001). On the same grounds, HSV-1 has been suggested to be a risk factor in other conditions like in Behcet's disease, Bell's palsy and Parkinson's disease (Hegab and Al-Mutawa, 2000; Simmons, 2002; Hemling *et al.*, 2003). Furthermore, HSV could also be a contributory cause of arteritis leading to atherosclerosis, an idea supported by some animal studies (Leinonen and Saikku, 2002).

In all cases where HSV is associated with chronic inflammatory lesions, experimental verification of a causative role is lacking.

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Persistence in the population: epidemiology, transmission

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Epidemiology of HSV-1 and HSV-2

Herpes simplex viruses are among the most ubiquitous of human infections. The frequency of HSV infection has been measured by testing various populations for the presence of antibody, as both virus and the immune response are thought to persist after infection for the life of the host. Worldwide, ~90% of people have one or both viruses. HSV-1 is the more prevalent virus, with 65% of persons in the United States having antibodies to HSV-1 (Xu *et al.*, 2002). The epidemiology in Europe is similar, with at least half of the population seropositive for HSV-1. In the developing world, HSV-1 is almost universal, and usually acquired from intimate contact with family in early childhood (Whitley *et al.*, 1998). After childhood, the HSV-1 prevalence rates increase minimally with age. Rates of HSV-1 infection are similar for men and women. In the United States, African-Americans and Asians have higher rates of HSV-1 infection than whites. The majority of infections are oral, although most are asymptomatic. Some data suggest that in developed countries, acquisition of HSV-1 is delayed from early childhood to adolescence or young adulthood (Hashido *et al.*, 1999; Mertz *et al.*, 2003).

HSV-2 infections are markedly less frequent than HSV-1 infections, with 15%–80% of people in various populations infected (Corey and Wald, 1999). The rates of infection vary with country as well as levels of sexual activity. In some countries, such as Spain and the Philippines, the HSV-2 prevalence hovers around 10%, increasing to 20%–30% range for most European countries and the United States (Varela *et al.*, 2001; Smith *et al.*, 2001; Enders *et al.*, 1998; Malkin *et al.*, 2002). Developing countries bear a much higher burden of HSV-2 infection, with many populations in Africa having >50% prevalence in the general population (Weiss *et al.*, 2001). Because HSV-2 infections are transmitted almost exclusively during sexual activity, the risk of HSV-2 reflects a person's level of sexual activity and the number of partners, and background prevalence of infection in the community. In communities with relatively low rates of infection, the risk of HSV-2 infection reflects more closely sexual activity of the person. However, in communities with high prevalence of infection, demographic rather than behavioral factors reflect HSV-2 risk more accurately (Sucato *et al.*, 2001; Rosenthal *et al.*, 1997; Austin *et al.*, 1999). Women have a greater risk of HSV-2 acquisition, reflecting both increased biologic susceptibility and pattern of relationships with older men, who are more likely to be HSV-2 seropositive. HSV-2 prevalence in the United States is higher among African-Americans than among whites and Asians (Fleming *et al.*, 1997). As a result, there is great disparity in infection rates according to both gender and race. For example, for white women, the risk of HSV-2 increases from about 18% among those with 2–4 lifetime partners to 35% for those with 10 to 49 lifetime partners (Fleming *et al.*, 1997). In contrast, for African-American women the risk increases steeply even with fewer partners, and exceeds 60% for women with more than 4 lifetime partners. For white men, the risk is ~10% among those who report 2 to 9 lifetime partners, and reaches 40% in those with >50 lifetime partners. Among African-American men, the risk rises from 35% in those with 2–4 lifetime partners to ~ 50% in those reporting >50 lifetime partners. The increase in the frequency of HSV-2 antibodies starts in adolescence, reflecting the initiation of sexual activity, and levels off in the 40s, probably reflecting cessation of new partner acquisition (Blower and Boe, 1993). In the United States, most people acquire HSV-2 in their 20s with a mean age at presentation of 24 years. In contrast, in South Africa, girls acquire HSV-2 infection in adolescence and >60% are infected by the age of 21 (Chen *et al.*, 2000).

The advent of the HIV epidemic initially eclipsed HSV-2 as a viral sexually transmitted disease of importance, but recent data have increasingly showed multiple interactions between the two viral infections (Corey *et al.*, 2004a). The development of molecular diagnostics has revealed that HSV-2 is the most common etiologic agent of genital ulcers in the developed and developing world (Chen *et al.*, 2000; Serwadda *et al.*, 2003). Even in regions in which syphilis and chancroid have been historically considered responsible for most genital ulcerations, the use of PCR-based techniques has clearly shown a predominance of HSV (Beyrer *et al.*, 1998; Morse *et al.*, 1997). In almost all studies, and in all populations, having HSV-2 infection increases the risk of HIV acquisition (Wald and Link 2002; Freeman *et al.*, 2006). The mechanism probably involves both HSV-2 induced skin or mucosal ulcerations, as well as influx of CD4+ cells into the herpetic lesions, cells that provide receptor for entry of HIV (Koelle *et al.*,1994). As transmission is more difficult to study than acquisition, the role of HSV-2 in the transmission of HIV is less well defined (Cameron *et al.*, 1989). However, the biology also suggests that HSV-2 infection may amplify HIV transmission, as HIV virions have been demonstrated in herpes ulcers (Schacker *et al.*, 1998c; Ballard, 2001). This topic of HSV and HIV interactions has been recently reviewed (Corey *et al.*, 2004a).

Spectrum of clinical disease

HSV can cause both mucocutaneous and systemic disease, and both HSV-1 and HSV-2 can cause the same syndromes, although the viruses are preferentially more likely to be associated with some syndromes than others. The variability in clinical expression is poorly understood, but the host immune system appears to be the main determinant of the clinical manifestations of HSV infections. The most severely affected are neonates, who usually acquire the disease during birth through exposure to infected genital secretions (Whitley *et al.*,1980). Rarely, adults can develop severe or fatal HSV infection during acquisition, and pregnant women appear to have a higher risk for this syndrome (Kobberman *et al.*, 1980; Sutton *et al.*, 1974). In most persons, HSV infections are confined to skin and mucosa. However, these can be severe, especially in persons immunocompromised either by other diseases (HIV, lupus), or iatrogenic immunosuppression or transplant, or extensive skin disease, such as eczema (Luchi *et al.*, 1995; Wheeler, Jr and Abele, 1966). Certain HSV-associated syndromes, such as HSV uveitis, have a strong immunopathogenic component and respond to immunosuppressive therapy (Balfour, 1994; Lairson *et al.*, 2003).

Immunocompetent host

Oral herpes

HSV-1 causes oral and labial, and occasionally facial, lesions. Initial infection is the most severe with ulcerative, painful stomatitis that usually occurs in children and is often associated with fever, anorexia and local edema of oral mucosa interfering with swallowing (Amir *et al.*, 1999). The lesions last a mean of 12 days and HSV-1 can be isolated in culture for the initial 7 days. The most common complication is dehydration requiring intravenous fluids, although secondary bacterial infection can also occur. In young adults, the presentation of initial oral HSV-1 infection can include pharyngitis, and tonsillectomy is occasionally (and erroneously) performed (Evans and Dick, 1964; Langenberg *et al.*, 1999).

Reactivation of HSV-1 in the mouth usually causes lesions on the lip ("fever blisters" or "cold sores"). The initial symptoms of pain, tingling, and itching occur prior to lesion appearance and are termed "prodrome" (Spruance, 1984). Initial lesion is an erythematous papule that evolves into a fluid-filled blister (Spruance *et al.*, 1997). Often there is a cluster of blisters, usually on a localized part of the lip, most often at the vermillion border. The lesions can also extend to skin on the face, and sometimes occur only on the face. The vesicles dry into a crust, and eventually re-epithelialize without scarring. The episodes last an average of 5 to 7 days. Only about 30% of persons with serologic evidence of HSV-1 have recurrent oro-labial herpes. Among those, 40% will have more than one recurrence per year. Known triggers of HSV-1 reactivation include facial trauma, surgery, fever and exposure to UV light (Spruance *et al.*, 1991). Oral labial HSV lesions were often associated with pneumococcal pneumonia and thought to be stimulated by the rapid rise in body temperature. Reactivation by UV light, such as occurs in skiers, can be abrogated by preventative use of sunscreen or antivirals (Spruance *et al.*, 1988). Recent popularity of laser skin resurfacing has been associated with severe HSV outbreaks resulting in recommendations that these procedures should be prophylaxed by antiviral therapy (Alster and Nanni, 1999; Beeson and Rachel, 2002). These triggers suggest that both systemic and skin factors can result in HSV reactivation (Hill *et al.*, 1978).

Genital herpes

HSV also causes ulcerations of genital mucosa and skin. The more common cause of genital herpes is HSV-2. However, recent studies suggest that 20%-50% of incident episodes

of genital herpes are caused by HSV-1 and the proportion of such incident cases due to HSV-1 may be increasing (Lafferty *et al.*, 2000; Lowhagen *et al.*, 2000; Vyse *et al.*,2000; Mertz *et al.*, 2003; Ross *et al.*, 1993). The reasons for this are not entirely clear but decreased HSV-1 acquisition in childhood and preferential practice of oral–genital sex during adolescence may be partly responsible. The clinical course of the initial and subsequent episodes is the same for both viruses; however, the frequency of recurrences and shedding is quite different, with HSV-1 reactivating infrequently in comparison to HSV-2 (Lafferty *et al.*, 1987; Benedetti *et al.*, 1994; Engelberg *et al.*, 2003). As such, it is important to identify the type of virus that causes the infection.

The severity of infection with HSV depends on previous immunity to HSV. Primary infection, defined as the first encounter with HSV-1 or HSV-2, is clinically most severe, and most likely to be symptomatic (Corey and Spear, 1986; Corey *et al.*, 1983). Non-primary infection is a new HSV-2 infection in a person with prior HSV-1 infection. New infections are diagnosed by detecting the virus on the mucosa in a person without concomitant antibody to the same type of virus. Recurrent infections occur as a result of reactivation of a previous, latent infection, and are identified by the presence of antibody at the time of initial presentation. It is important to note that accurate classification of an episode must include both virologic information as well as determination of antibody status because there is a wide overlap in clinical manifestations of the infection. Although primary infection is more likely to be symptomatic than an episode of reactivation, only up to 39% of people who acquire primary HSV will be diagnosed with the infection at that time (Langenberg *et al.*, 1999). A substantial proportion will become symptomatic at some point during the disease and present with a first clinical episode of genital herpes (Bernstein *et al.*, 1984; Diamond *et al.*, 1999). In a recent study of 401 persons presenting with a first episode of genital herpes, 91 (23%) had primary infection with HSV-1, 139 (35%) had primary infection with HSV-2, 36 (9%) had non-primary initial HSV-2 and 135 (37%) had a first recognized recurrence of HSV-2.

The painful genital vesicles and ulcers accompanied by inguinal adenopathy and systemic flu-like illness are part of the classic presentation of first episode of genital herpes (Corey *et al.*, 1983). The evolution of lesions is similar to those of oral herpes, usually with more rapid progression to ulcers in women, and often a prolonged vesicular phase in men. The lesions are widely distributed in the genital area, and multiple (up to 100 lesions) can be seen. During an initial episode, lesions last up to 3 weeks, and new lesion formation continues for 10–14 days. Itching, tingling and pain can be severe. Neurologic complications, such as meningitis and bladder paresis, usually transient, occur in ~10% and are more common among women. External dysuria is also common among women. Proctitis is common among MSM and can be associated with transient bowel dysfunction (Quinn *et al.*, 1981).

Recurrent episodes of genital HSV-2 occur a median of 4 (women) to 5 (men) times during the first year (Benedetti *et al.*, 1994). However, there is great variability in the frequency of recurrences, even during the first year. In a study of 457 persons with newly acquired HSV-2 infection, 38% had 6 or more recurrences and 20% had more than 10 recurrences during the first year. 14% of women and 26% of men had more than 10 recurrences and only 26% of women and 8% of men had no or 1 recurrence in the first year of infection (Benedetti *et al.*, 1994). Subsequently, the frequency of episodes slowly decreases, with an average decrease of 2 recurrences between years 1 and 5 of infection. As such, most patients will not perceive the decrease in severity until several years have elapsed. This improvement is not universal, and some people will continue to have very frequent or even more frequent recurrent episodes many years into the infection. In contrast, HSV-1 infection recurs infrequently, with a median of 195 days to first recurrence among women and 567 days among men after documented new genital HSV-1. Subsequently, the rate of recurrences falls even further with only 19% having 1 recurrence, and 15% having two or more recurrences during the second year after genital HSV-1 infection (Engelberg *et al.*, 2003).

Genital herpes is often associated with psychosocial distress, caused by having an incurable STD, stigma of having such disease, and anxiety about resuming normal sexual life after acquisition (Catotti *et al.*, 1993; Carney *et al.*, 1994; Swanson and Chenitz, 1990). The distress is usually greater among women than men and in many persons it surpasses the physical discomfort caused by the infection. Over time, most people adjust to living with herpes, although recurrences of depression and feelings of worthlessness tend to return during recurrences. Oral herpes can also be associated with feelings of being damaged, as it is cosmetically more obvious; however, it is clearly associated with less social stigma.

Other mucocutaneous infections

Despite the common involvement of oral or genital mucosa in the acquisition of HSV, cutaneous infections at other body sites are also well recognized. Eczema herpeticum occurs occasionally in persons with atopic dermatitis, regardless of whether they are receiving topical steroids (Wollenberg *et al.*, 2003; Yoshida and Amatsu,

2000). Outbreaks of herpes gladiatorum occur among young athletes involved in contact sports, often high-school wrestlers (Anderson, 2003; Becker,1992; Belongia *et al.*, 1991). Both infections are usually caused by HSV-1, and respond to therapy with antiviral medication (Niimura and Nishikawa,1988; Anderson, 1999).

Herpetic whitlow results from infection of the distal finger with HSV. Historically, this disease was caused by HSV-1 and was acquired among dental or nursing professionals (Stern *et al.*, 1959; Manzella *et al.*, 1984). More recently, with the adoption of universal precautions, the incidence of HSV-1 whitlow has decreased, and most distal finger infections arise in the setting of primary genital HSV-2 infections.

Recent studies have shown a link between erythema multiforme and recurrent HSV infections (Huff *et al.*, 1983). While the pathogenesis is not completely understood, strong association of erythema multiforme with particular HLA-DQ alleles is consistent with an immunopathologic basis (Malo *et al.*, 1998; Kampgen *et al.*, 1988). Molecular studies of the involved skin have demonstrated HSV DNA in the erythema multiforme lesions, and reports of prevention of attacks with oral acyclovir support HSV as an etiologic factor in this disease (Brice *et al.*, 1989; Miura *et al.*, 1992; Ng *et al.*, 2003; Lemak *et al.*, 1986).

HSV infection in CNS

Reactivation of HSV in the CNS is associated with 2 distinct syndromes with vastly different prognoses. Recent studies have shown that recurrent benign meningitis, or Mollaret's meningitis, results from HSV infection (Cohen *et al.*, 1994; Picard *et al.*, 1993). Most often HSV-2 is implicated, although HSV-1 has also been reported (Yamamoto *et al.*, 1991). Women are at higher risk for this complication than men, and often develop the initial episode during acquisition of genital HSV-2, with subsequent recurrent episodes. Thus, the epidemiology of Mollaret's meningitis parallels that of genital herpes. However, not infrequently the meningitis is the presenting complaint, and the association with HSV-2 is not always recognized. Spinal fluid findings are consistent with "aseptic meningitis" with a lymphocyte predominance, fairly normal protein and glucose, and sterile fluid. HSV DNA can be detected by PCR (Yamamoto *et al.*, 1991; Cohen *et al.*, 1994). While unpleasant, this condition is benign, and anecdotal data suggest that individual episodes respond well to antiviral therapy and further episodes can be abrogated in large part by suppressive antiviral therapy. In contrast, HSV encephalitis is a disease of severe morbidity (Whitley and Lakeman 1995). The usual agent is HSV-1, although HSV-2 meningoencephalitis has also been described in immunosuppressed patients (Gateley *et al.*, 1990; Linnemann *et al.*, 1976). HSV encephalitis is the most common cause of sporadic encephalitis in adults, with an estimated frequency of 1 in 200,000 to million persons. There is no gender predilection, and the age distribution appears bimodal, with a smaller peak among youth and a larger peak among the elderly. Encephalitis can develop both during primary infection (usually among younger people) and during reactivation (usually among older people) of HSV. Classically, the patient presents with fever and signs of focal encephalitis, such as seizures, headache and focal neurologic deficits. However, the initial symptoms can be insidious and include personality and cognitive disturbances. Fever is common. Spinal fluid shows increased white count, usually but not always with lymphocyte predominance, and can be bloody with abnormal chemistry. Imaging studies are not pathognomic, although temporal lobe disease is typical. The diagnosis should always be confirmed virologically. Most cases are diagnosed with the use of PCR that has surpassed the "gold standard" of brain biopsy because of similar sensitivity but virtually no risk (Lakeman and Whitley, 1995; Puchhammer-Stockl *et al.*, 1993). However, lack of positive PCR in the spinal fluid does not rule out the diagnosis of HSV, especially early in the disease, and intravenous acyclovir should be initiated if the clinical picture is compatible, no alternative diagnosis is made, and the PCR is negative (Whitley *et al.*, 1986). The lumbar puncture should be repeated in 24–48 hours and CSF submitted for PCR testing again. Untreated HSV encephalitis has >70% fatality rate. Even with therapy, HSV encephalitis results in death in a substantial proportion of patients and only a few percent return to normal function.

Eye disease

Occasionally, oral HSV-1 infection is associated with blepheritis or conjunctivitis (Souza *et al.*, 2003). While these are benign manifestations of herpetic eye infection, herpetic keratitis causes significant morbidity (Liesegang, 2001). Clinically, the disease is manifested by pain, photophobia and visual impairment. Dendritic ulcers can be visualized on examination with fluorescein staining. Recurrent episodes of reactivation are associated with stromal involvement and lead to progressive loss of vision and scarring, requiring penetrating keratoplasty or corneal transplants. Since the onset of keratitis is rarely coincidental with initial acquisition of HSV infection, the corneal infection may either result from direct inoculation of the virus into the eye, or more likely, from reactivation of HSV in the distribution that enervates the eye. The predisposition to

herpes keratitis is not well understood but the infiltrate of HSV-specific T lymphocytes supports the immunopathologic basis for this disease (Deshpande *et al.*, 2001; Koelle *et al.*, 2000; Thomas *et al.*, 1997; Thomas and Rouse, 1998; Verjans *et al.*, 2000). The complications of HSV-1 keratitis are the leading cause of infectious blindness in the United States (Lairson *et al.*, 2003). Clinical trials have demonstrated the benefit of suppressive acyclovir in the prevention of herpetic keratitis recurrences (Herpetic Eye Disease Study Group 1997, Wilhelmus *et al.*, 1998).

Acute retinal necrosis is another HSV-related syndrome that often results in blindness. The pathogenesis is poorly understood, and HSV-2 is detected more often than HSV-1 in this disease (Itoh *et al.*, 2000; Thompson *et al.*, 1994; Tran *et al.*, 2004). Immunosuppression appears to be a risk factor, as the acute retinal necrosis appears to be more common among patients with AIDS, although this syndrome has also been observed in persons with iatrogenic immunosuppression (Guex-Crosier *et al.*, 1997). The presentation is often rapid, and loss of sight is frequent. Antiviral therapy may prevent involvement of the contralateral eye, even if it does not restore vision in the affected eye (Tran *et al.*, 2004).

Other syndromes

Other, infrequent manifestations of HSV have also been reported. Of note is disseminated HSV, which occurs occasionally in persons who appear immunocompetent and has a high fatality rate (Goyette and Donowho, 1974; Flewett *et al.*, 1969; Keane *et al.*, 1976; Frederick *et al.*, 2002; Chase *et al.*, 1987). While the infection most likely begins as oral or genital herpes, these localized symptoms are often not recognized, and patients present with fulminant hepatitis with transaminases in the thousands, diffuse rash, or other systemic manifestations. Death results from sepsis with DIC, ARDS, or progressive hepatic failure. This syndrome occurs more frequently among women in the second half of pregnancy although occasional cases are reported among nonpregnant women and men. Factors predisposing to this have not been described. Early administration of acyclovir is often effective, but the disease is often not diagnosed premortem.

Neonatal herpes

The frequency of neonatal herpes varies by region and is estimated to occur from 1 in 3200 to 1 in 15 000 pregnancies (Sullivan-Bolyai *et al.*, 1983a; Tookey and Peckham, 1996; Mindel *et al.*, 2000; Brown *et al.*, 2003; Gutierrez *et al.*, 1999). Reasons for the variant frequency are poorly understood but are likely to result from interplay between sexual behavior in the population and the baseline prevalence and incidence of HSV-1 and HSV-2. Over 85% of neonatal herpes is acquired from intrapartum exposure of the newborn to infected maternal secretions. In 5% of cases, congenital infection of the fetus in the setting of new acquisition of HSV during pregnancy has been reported (Florman, 1973; Sullivan-Bolyai *et al.*, 1983a). These infants are born with clinical evidence of disseminated disease, often including skin lesions, may be premature and have a poor prognosis. Post-natal acquisition of HSV, often from non-maternal sources, has also been reported in about 10% of cases, and is associated with HSV-1 infection.

Recent prospective studies have clarified risk factors for HSV transmission during delivery (Brown *et al.*, 1997; Arvin *et al.*, 1986; Prober *et al.*, 1992). The greatest risk of neonatal herpes is conferred by viral shedding, defined as HSV isolation in maternal genital secretions at the time of parturition, with a relative risk of neonatal HSV of >300 compared with women who do not have HSV isolated during labor (Brown *et al.*, 2003). However, some infants acquire neonatal herpes despite lack of culturable virus at the time of delivery. In some of these cases, HSV DNA can be detected by PCR despite negative viral culture, suggesting that culture can be falsely negative. Of greater concern is the observation that only 5% of women who have HSV isolated from the genital tract at the time of delivery transmit HSV to the infant. As such, these women are at potential risk of unnecessary interventions.

Among women who are shedding HSV in genital secretions at labor, risk factors for neonatal herpes include newly acquired HSV infection (RR = 59), cervical vs. vulvar viral isolation (RR = 15), young mother (RR = 2.7 for women aged <21), and HSV-1 vs. HSV-2 isolation (RR = 35). Cesarean deliveries appear protective as women who are delivered abdominally had a significantly lower risk of HSV transmission compared with women who had vaginal delivery (RR = 0.14). Because even the largest series of neonatal herpes contain only a handful of cases, the exact contribution of each risk factor is difficult to measure. However, overall, most cases of neonatal herpes appear to occur among women who acquire subclinically new genital HSV-1 or HSV-2 and who deliver vaginally (Arvin *et al.*, 1982, Whitley *et al.*, 1991b). In one study, 4 of 9 women who acquired HSV so late in pregnancy that they did not seroconvert by the time of delivery transmitted the virus to their infant (Brown *et al.*, 1997). Management of recognized newly acquired genital herpes at the end of pregnancy needs to be individualized and should include consideration of administration

of acyclovir to women toward the end of pregnancy, scheduled abdominal delivery prior to rupture of membranes, and prophylactic antiviral therapy of the newborn (Prober *et al.*, 1992; Sheffield *et al.*, 2003).

Infants with neonatal herpes often present with nonspecific complaints such as fever, fussiness, sepsis, or seizures (Whitley *et al.*, 1998). Typical skin lesions are not noted universally, and depending on the disease classification, may develop only in up to 80% of patients. Clinically, neonatal herpes has been divided into 3 syndromes. In recent studies, skin, eye and mouth disease accounted for 42% of cases, and is defined by disease that is present only on skin or mucosa (Whitley *et al.*, 1998, Kimberlin *et al.*, 2001a). This form of infection has the best prognosis with negligible mortality and up to 70% of treated infants having normal development. Of interest, even those infants who did not have any evidence of CNS involvement may subsequently present with neurologic deficits, suggesting that subclinical and/or delayed involvement of the brain is not uncommon. Disseminated disease accounts for 23% of cases and has the highest mortality (60% with therapy). Among the survivors, normal development is noted in about 60%. CNS disease comprises the remaining 35% of newborns with neonatal herpes. Mortality is low, but this form of disease is associated with highest morbidity as less than 50% will have normal development. Comparison of secular trends suggests that a greater proportion of cases are diagnosed with SEM disease in recent years compared with an earlier cohort (Whitley *et al.*, 1988, 1991a, b). A potential explanation is that the diagnosis is made earlier, prior to dissemination or CNS invasion. Many cases are still diagnosed late, or post-mortem, and administration of acyclovir to infants with sepsis-like syndrome is not universally done. Prompt antiviral therapy is associated with decreased morbidity and mortality, but the prognosis remains grave for most children. HSV-2 appears more neuroinvasive in newborns than HSV-1, and as such, has a worse prognosis (Corey *et al.*, 1988).

The observations about risk factors for neonatal herpes suggest that prevention of neonatal herpes relies on prevention of HSV acquisition in late pregnancy. This strategy, in turn, relies on identification of women at risk for HSV infection with the use of type-specific serology, and, potentially, the serologic testing of their sex partners. This approach has not been widely accepted (Wilkinson *et al.*, 2000; Brown, 2000). Reasons for resistance are numerous, including lack of confidence in the performance of commercial type-specific serologies, perception that counseling about results of HSV serologies is burdensome to both providers and patients, lack of simple interventions, and the relative rarity of neonatal HSV. Of note, after institution of universal testing for Group B streptococcus (GBS) during the last trimester, the frequency of neonatal GBS sepsis now approaches that of neonatal HSV (Gibbs *et al.*, 1994; Chuang *et al.*, 2002; Schrag and Schuchat, 2004).

Immunocompromised persons

Immunosuppression, regardless of etiology, is associated with greater risk of HSV reactivation, prolonged viral shedding and more severe clinical recurrences (Meyers *et al.*, 1980; Siegal *et al.*, 1981). While even in severely immunocompromised patients most disease is mucocutaneous, extension to internal organs, such as esophagus, or dissemination, can also occur. Other syndromes include hepatitis and pneumonia (Ramsey *et al.*, 1982). Patients receiving cancer chemotherapy are at risk for HSV recurrences during periods of neutropenia; the risk in organ or marrow transplant patients is prolonged and parallels the duration of immunosuppression (Wade *et al.*, 1984a). However, the greatest risk of HSV reactivation after bone marrow transplant occurs early during the initial neutropenia associated with myeloablation. This is in contrast to other herpesvirus infections, such as VZV and CMV, which tend to occur later during the post-transplant period during maximal suppression of cell-mediated immunity. Because HSV has a significant impact on post-chemotherapy and post-transplant morbidity, acyclovir prophylaxis is administered routinely in this population (Wade *et al.*, 1984b).

In the last two decades, HIV has emerged as the most common cause of immunosuppression worldwide. Not surprisingly, early clinical reports of patients with HIV document extensive clinical HSV recurrences (Siegel *et al.*, 1988). The disease burden is especially great, as persons at risk for sexually transmitted HIV are more likely to have HSV-2 infection than the general population. Prior to the introduction of effective antiretroviral therapy, chronic HSV ulcers accounted for a small proportion of newly diagnosed persons with AIDS, and developed in many other persons as immunosuppression progressed. Despite the frequent presence of HSV-1 and HSV-2 infection (90% overall) in patients with HIV, extensive clinical disease develops only in a minority of patients (Bagdades *et al.*, 1992). The immunologic and virologic risk factors for developing severe disease are not understood. Systematic study of clinical and virologic aspects of genital HSV-2 shows that even in the absence of overt clinical disease, HIV infected persons have high rates of viral shedding. Among a group of 68 men who have sex with men with HIV infection and a

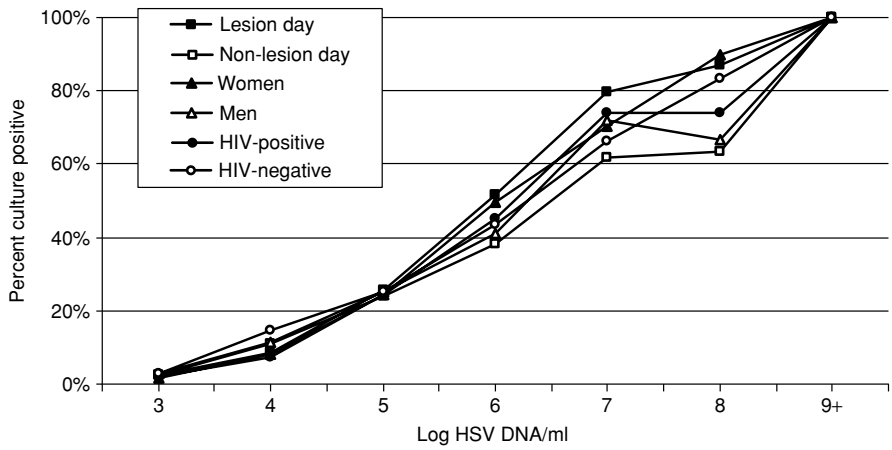

Fig. 36.1. The relationship between the probability of HSV isolation in viral culture and the number of copies of HSV DNA as detected by PCR (adapted from Wald (Wald *et al.*, 2003)).

mean CD4 count of 351, the rate of genital HSV isolation was 9.7% of days, and the perianal area accounted for 79% of isolates (Schacker *et al.*, 1998b). The relative risk of total viral shedding was elevated at 3.3 compared with men who are HIV negative, but an even greater relative risk of 6.9 was shown for subclinical shedding. These observations show that HIV has a greater effect on the virologic than clinical aspects of the natural history of HIV and may provide an explanation for a burgeoning epidemic of HSV-2 in parallel with HIV in sub-Saharan Africa.

Immune reconstitution with antiretroviral therapy (ART) has resulted in a decrease in risk of several opportunistic infections, and has allowed for stopping of prophylaxis in those patients with vigorous CD4 response. Unfortunately, the data suggest that the increase in CD4 cells associated with ART prevents lesions, with significantly lower risk of mucocutaneous lesions among patients treated with ART compared with patients who are untreated, but has a negligible effect on viral shedding (Posavad *et al.*, 2001). As such, HSV-2 & HIV seropositive patients are likely to continue to be infectious for HIV and HSV-2 and suppressive HSV therapy should be considered in that setting.

Viral shedding

HSV is present intermittently on skin or mucosa in between symptomatic recurrences. This phenomenon, defined as asymptomatic or subclinical shedding, has been described since the early clinical descriptions of genital herpes. However, the frequency, pattern, and the importance of subclinical shedding for transmission of HSV have only recently been elucidated.

The frequency of viral shedding has been measured both by culture and by PCR. Studies using amplification techniques show that HSV DNA PCR is up to 400% more sensitive for detection of HSV on mucosal surfaces than viral isolation (Wald *et al.*, 2003) (Fig. 36.1). The frequency of viral shedding varies with type of HSV, duration of infection, gender, and immune status. Most variability observed in the frequency of viral reactivation is not explained by these risk factors, suggesting that there is a strong host immunogenetic (or viral strain) factor in determining the severity of disease.

The initial study examining prospectively viral shedding among women with recent genital HSV-2 infection showed that HSV was detected by PCR on 28% of days sampled (Wald *et al.*, 1997). However, as shown in Fig. 36.2, the variability in the frequency of reactivation is great, even among this homogeneous group of women. Subsequent studies have also examined viral shedding among men. In a group of men with either recent acquisition of genital HSV-2 or a history of frequent recurrences, the overall rate of HSV detection from the genital area by PCR was 32% (median 30%; range 0 to 92%) (Wald *et al.*, 2002b). Despite the rough parallel between the frequency of viral shedding and the frequency of recurrent lesions, these two processes are somewhat independent, with some persons having frequent days with lesions, or prolonged lesions, and others having frequent viral shedding but without many recurrences. These observations suggest that the immunologic mechanisms that control viral shedding may differ from those that control lesion formation and resolution. Animal experiments, and findings in persons with immune compromise, indicate that the CD4 response predominates in control of lesions, while CD8 response is more important for control of viral reactivation (see Chapter 34).

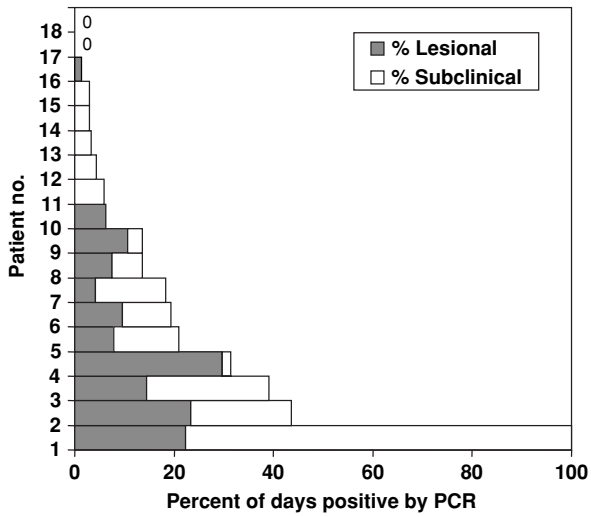

Fig. 36.2. Variability in symptomatic and subclinical shedding among 26 women with genital HSV-2 infection for less than 2 years.

Transmission dynamics

Figure 36.3 illustrates the pattern of viral shedding during inadvertent sexual transmission of HSV-2 infection. The woman participating in a daily home sampling study had symptomatic genital HSV-2 infection for 3.5 years, and had initiated a new relationship with a partner who was HSV seronegative. Despite having occasional non-specific symptoms, she did not notice a recurrence during the episode of subclinical viral shedding that resulted in transmission of HSV-2. Studies consistently indicate that transmission to sex partners, or to neonates, usually occurs during such episodes of subclinical shedding (Barton *et al.*, 1987; Mertz *et al.*, 1992).

Prospective studies of HSV-2 discordant couples have been used to estimate rate of transmission and ascertain risk factors for transmission. Unfortunately, only a few such studies have been done that included a sufficiently large number of persons to obtain reliable estimates of rates of transmission and risk factors (Mertz *et al.*, 1988, 1992; Stanberry *et al.*, 2002; Bryson *et al.*, 1993; Wald *et al.*, 2001; Corey *et al.*, 2004b). The rate of HSV-2 acquisition among persons at risk varies from a high of 8.6 per 100 person-years for women, to a low of 2.7 per 100 person-years among men. These studies have also shown that (1) women are at 2-to-6 fold higher risk for HSV-2 acquisition than men; (2) prior infection with HSV-1 does not protect against HSV-2 acquisition; (3) symptoms of first episode HSV-2 infection are less prominent among those with previous HSV-1 infection; (4) frequent sexual activity is a risk factor for HSV-2 transmission, and (5) HSV-2 is transmitted more easily than HSV-1 infection. Other characteristics that have been associated with increased risk of HSV-2 transmission, but inconsistently, or without reaching statistical significance, include short duration of relationship and short duration of genital herpes in the source partner prior to study participation, and sex during recurrences (Corey *et al.*, 2004b). Condoms appear to be protective, but the degree of protection afforded by consistent use, and effect of condom use on female-to-male vs. male-to-female transmission varies among the studies (Wald *et al.*, 2001). In addition, the use of condoms in monogamous, long-term relationships is rare, even in the settings of known HSV-2 discordance and extensive counseling in the context of a clinical trial. Available data indicate that consistent condom use offers partial protection ($\sim$ 50%) against HSV-2 acquisition at best. A recent study of daily suppressive valacyclovir has shown that transmission is decreased by 48% among those couples who were randomized to receive antiviral therapy (Corey *et al.*, 2004b). These results offer couples another option to use to decrease the risk of HSV-2 transmission, and are an added benefit to the use of daily antiviral therapy (see below).

Management and Prevention

Treatment

The advent of antiviral drugs for HSV-1 and HSV-2 infections has made clinical management of these infections a part of standard clinical practice (Table 36.1). For mucocutaneous and visceral HSV infections, acyclovir and its related compounds famciclovir and valacyclovir have been the mainstay of therapy (Whitley and Gnann Jr, 1992). Several antiviral agents are available for topical use in HSV eye infections: idoxuridine, trifluorothymidine and topical vidarabine. For HSV encephalitis and neonatal herpes, intravenous acyclovir is the treatment of choice. Acyclovir resistant virus can be encountered in immunocompromised hosts (Erlich *et al.*, 1989; Reyes *et al.*, 2003).

Acyclovir was the first antiviral clearly demonstrated to be effective against HSV infections (Elion *et al.*, 1977). It is an acyclic nucleoside analogue that is a substrate for HSV-specific thymidine kinase. Acyclovir is selectively phosphorylated by HSV-infected cells to acyclovir-monophosphate. Cellular enzymes then phosphorylate acyclovir-monophosphate to acyclovir-triphosphate, a competitive inhibitor of viral DNA polymerase. Acyclovir-triphosphate is incorporated into the growing DNA chain of the virus and causes chain termination. Acyclovir has potent in vitro activity against both HSV-1 and HSV-2.

Valacyclovir is the valyl ester of acyclovir and is metabolized in the gut, liver and epithelium to acyclovir and

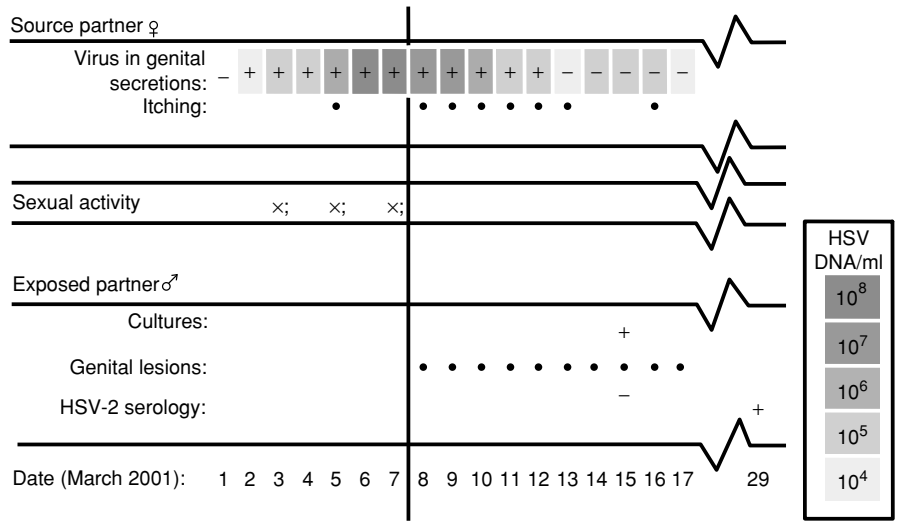

Fig. 36.3. Sexual transmission of HSV-2 during subclinical shedding.

produces much higher levels of drug leading to more convenient therapy (Soul-Lawton *et al.*, 1995). Famciclovir, the oral formulation of penciclovir, is also clinically effective in the treatment of a variety of HSV-1 and HSV-2 infections (Pue and Benet, 1993). Ganciclovir has activity against both HSV-1 and HSV-2, but because it is more toxic than acyclovir, valacyclovir, and famciclovir, it is generally not recommended for treatment of HSV infections. Numerous trials of acyclovir in mucocutaneous HSV infections of immunocompetent and immunosuppressed host have been conducted. General recommendations are outlined below (Centers for Disease Control and Prevention 2002). Increasingly, shorter courses of therapy are being utilized for treatment of recurrent mucocutaneous HSV-1 or HSV-2 in immunocompetent patients.

Treatment of recurrent mucocutaneous herpes

Among immunocompetent patients, recent studies have shown the effectiveness of short course therapy to reduce the signs and symptoms of oral and genital HSV infection. These include 2 g twice daily for one day of valacyclovir for oral-labial HSV and 2 and 3 day courses of acyclovir or valacyclovir for recurrent episode genital herpes (Spruance *et al.*, 2003; Leone *et al.*, 2002; Wald *et al.*, 2002a). One-day therapy with famciclovir for genital herpes also appears to increase the probability of an aborted recurrence and to shorten the duration of lesions and symptoms, compared with placebo (Aoki *et al.*, 2006).

Suppression of mucocutaneous herpes

Recognition of the high frequency of subclinical reactivation has provided increasing rationale for the use of daily antiviral therapy to suppress reactivations of HSV. This is especially useful for persons with frequent clinical reactivations such as those with recently acquired genital HSV infection. Immunosuppressed persons, including those with HIV infection, may also benefit from daily antiviral therapy. A variety of dosages have been utilized.

Reduction in transmission to sexual partners

Once daily valacyclovir (500mg) has been shown to reduce transmission of HSV in partnerships in which one partner has symptomatic genital HSV-2 and the other partner is susceptible (Corey *et al.*, 2004b). Serologic screening can be used to identify at risk couples, as many couples identified as discordant by history are concordant on serologic evaluation.

Severe HSV infection

Intravenous acyclovir (30 mg/kg/day, given as a 10 mg/kg infusion over 1 hour at 8-hour intervals) is effective in reducing the morbidity and mortality from HSV encephalitis (Whitley, 1988; Whitley and Lakeman, 1995). Early initiation of therapy is a critical factor in outcome. The major side effect associated with intravenous acyclovir is transient renal insufficiency, usually caused by crystallization of the compound in the renal parenchyma. This adverse reaction can be avoided if the medication is given slowly over 1 hour and the patient is well hydrated. Because CSF levels of acyclovir average only 30% to 50% of plasma levels, the dosage of acyclovir used for treatments of CNS infection (30 mg/kg per day) is double that used for the treatment of mucocutaneous or visceral disease (15 mg/kg per day). For neonatal HSV, high-dose intravenous therapy is

Table 36.1. Treatment of HSV infections

Infections in immunosuppressed patients

Acute symptomatic first or recurrent episodes: IV acyclovir (5 mg/kg q 8 h), or oral acyclovir (400 mg qid), famciclovir (500 mg po tid) or valacyclovir (500 mg po bid). Treatment duration may vary from 7 to 14 days.

Suppression of reactivation disease: IV acyclovir (5 mg/kg q 8 h), valacyclovir (500 mg po bid) or oral acyclovir (400–800 mg 3–5 times per day) prevent recurrences during the immediate 30 day post transplantation period. Longer term suppression is often used for persons with continued immunosuppression. In bone marrow and renal transplant patients, valacyclovir 2 grams 4 times daily is also effective in preventing CMV infection (Dignani *et al.*, 2002, Lowance *et al.*,1999). Valacyclovir 8 gm daily has been associated with thrombotic microangiopathy after extended use in HIV positive persons (Bell *et al.*, 1997). In HIV-infected persons, oral famciclovir (500 mg bid) is effective in reducing clinical and subclinical reactivations of HSV-1 and 2, and valacyclovir 500mg bid decreases the frequency of genital HSV-2 recurrences (Schacker *et al.*, 1998a, Romanowski *et al.*, 2000, DeJesus *et al.*, 2003).

Genital herpes

First episodes: Oral acyclovir (200 mg 5 times per day or 400 mg tid), oral valacyclovir (1000 mg bid) or famciclovir (250 mg bid) for 10–14 days are effective. IV acyclovir (5 mg/kg q 8 h for 5 days) is given for severe disease or neurologic complications such as aseptic meningitis.

Symptomatic recurrent genital herpes: Oral acyclovir (200 mg 5 times per day for 5 days, 800 mg po tid for 2 days), valacyclovir (500 mg bid for 3 or 5 days) or famciclovir (125 mg bid for 5 days). All these therapies are effective in shortening duration of lesions, viral shedding and symptoms.

Suppression of recurrent genital herpes: Oral acyclovir (200-mg capsules bid or tid, 400 mg bid, or 800 mg qd), famciclovir (250 mg bid), or valacyclovir (500 mg or 1000 mg qd or 500 mg bid) prevents symptomatic reactivation. Persons with frequent reactivation (<9 episodes) can take 500 mg daily; those with >9 should take 1000 mg/daily or 500 mg bid (Reitano *et al.*, 1998).

Oral-labial HSV infections:

First episode: Oral acyclovir (200 mg) is given 4 or 5 times per day. Famciclovir (250 mg bid) or valacyclovir (1000 mg bid) has been used clinically.

Recurrent episodes: Valacyclovir 1000 mg bid for 1 day or 500 mg bid for 3 days is effective in reducing pain and speeding healing. Self-initiated therapy with 6 times daily topical penciclovir cream is effective in speeding the healing of oral-labial HSV, topical acyclovir cream has also been shown to speed healing (Spruance *et al.*, 1997).

Suppression of reactivation of oral-labial HSV: Oral acyclovir (400 mg bid), if started before exposure and continued for the duration of exposure (usually 5–10 days), will prevent reactivation of recurrent oral-labial HSV infection associated with severe sun exposure (Spruance *et al.*, 1988).

Herpetic whitlow: Regimens used for treating genital herpes can be utilized but clinical trial data are lacking.

HSV proctitis: Oral acyclovir (400 mg 5 times per day) is useful in shortening the course of infection (Rompalo *et al.*, 1988); less frequent dosing is also likely to be effective. In immunosuppressed patients or in patients with severe infection, IV acyclovir (5 mg/kg q 8 h) may be useful.

Herpetic eye infections: In acute keratitis, topical trifluorothymidine, vidarabine, idoxuridine, acyclovir, penciclovir, and interferon are all beneficial. Debridement may be required; topical steroids may worsen disease.

CNS HSV infections

HSV encephalitis: Intravenous acyclovir (10 mg/kg q 8 h; 30 mg/kg per day) for 10 days is preferred.

HSV aseptic meningitis: No studies of systemic antiviral chemotherapy exist. If therapy is to be given, IV acyclovir (15–30 mg/kg per day) should be used in severely affected patients, followed by oral course of valacyclovir.

Autonomic radiculopathy: No studies are available.

Neonatal HSV infections: Acyclovir (60 mg/kg per day, divided into 3 doses) is given. The recommended duration of treatment is 21 days. Monitoring for relapse should be undertaken and some authorities recommend continued suppression with oral acyclovir suspension for 3 to 4 months (Kimberlin *et al.*, 1996).

Visceral HSV infections

HSV esophagitis: IV acyclovir (15 mg/kg per day). In some patients with a milder degree of immunosuppression, oral therapy with valacyclovir or famciclovir is effective.

HSV pneumonitis: No controlled studies exist. IV acyclovir (15 mg/kg per day) should be considered.

Disseminated HSV infections: No controlled studies exist. Intravenous acyclovir nevertheless should be tried, and in some cases has been reported to result in survival.

recommended (60 mg/kg per day in three divided doses) (Kimberlin *et al.*, 2001b). Intravenous therapy for neonatal herpes should be given for 21 days. Increasingly, serial testing of CSF HSV DNA has been utilized to guide the duration of therapy, and most experts advocate treating until HSV DNA is no longer detected. In immunosuppressed patients, IV acyclovir or oral valacyclovir are utilized to prevent HSV reactivations during transplantation or chemotherapy, and high doses of oral valacyclovir also prevent CMV reactivation (Dignani *et al.*, 2002; Lowance *et al.*, 1999).

Acyclovir-resistant strains of HSV have been identified, especially in HIV-infected persons. Almost all clinically significant acyclovir resistance has been seen in immunocompromised patients. Most acyclovir-resistant strains of HSV have a deficiency in thymidine kinase, the enzyme that phosphorylates acyclovir (Darby *et al.*, 1981). Thus, cross-resistance to famciclovir is usually found. Occasionally, an isolate with altered thymidine kinase specificity will arise and will be sensitive to famciclovir but not to acyclovir. In some patients infected with thymidine kinase–deficient virus, higher doses of acyclovir are associated with clearing of lesions. In others, clinical disease progresses despite high-dose therapy. Isolation of HSV from persisting lesions despite adequate dosages and blood levels of acyclovir should raise the suspicion of acyclovir resistance (Safrin *et al.*, 1992, 1994). In such cases therapy with the antiviral drug foscarnet is useful (Safrin *et al.*, 1991). Because of its toxicity and cost, this drug is usually reserved for patients with extensive mucocutaneous infections. Cidofovir is a nucleotide analogue (Snoeck *et al.*, 1994). Most thymidine kinase–deficient strains of HSV are sensitive to cidofovir. Cidofovir ointment has been shown to speed healing of acyclovir-resistant lesions (Lalezari *et al.*, 1997). Similarly, trifluorothymidine ointment has also been reported to be of utility (Birch *et al.*, 1992).

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Part III

Pathogenesis, clinical disease, host response, and epidemiology: alphaherpes viruses VZV

Ann Arvin and Richard Whitley

37

VZV: pathogenesis and the disease consequences of primary infection

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Introduction

VZV is a human alphaherpesvirus that causes varicella (chickenpox) as the primary infection and establishes latency in sensory ganglia. VZV reactivation results in herpes zoster (shingles). During the course of varicella and zoster, VZV infects differentiated human cells that exist within unique tissue microenvironments in humans. The tropism of VZV for skin is the most obvious clinical manifestation of VZV infection, producing the vesicular cutaneous lesions that are associated with varicella and zoster. The site of initial VZV infection in naïve hosts is thought to be mucosal epithelial cells of the upper respiratory tract. Entry is presumed to follow inoculation of the respiratory epithelium with infectious virus transmitted by aerosolized respiratory droplets or by contact with virus in varicella or zoster skin lesions (Arvin, 2001a; Grose, 1981). VZV in respiratory or conjunctival mucosal cells has the opportunity to interact with and infect local immune system cells and those in adjacent lymphoid tissues. Trafficking of infected peripheral blood mononuclear cells (PBMC), which appear to be predominantly T-cells, to the skin is thought to give rise to crops of cutaneous vesicles. Skin lesions contain VZV material associated with necrotic debris and, unlike virus grown in vitro, cell-free, infectious particles are detected in vesicular fluid (Williams *et al.*, 1962). The life cycle of VZV is completed upon its transmission to a susceptible host from an individual with varicella, or it can be postponed for decades by establishing latency in neurons and transmitting to future generations during episodes of zoster.

VZV shares its tropism for epithelial tissues with its relatives, HSV-1 and HSV-2, as well as with the non-human alphaherpesviruses. VZV also shares the neurotropism of these viruses, as discussed elsewhere in this volume. However, VZV seems to be more akin to the betaherpesviruses, HHV6 and HHV7, in its apparent tropism for T-cells (Ku *et al.*, 2002, 2004; Takahashi *et al.*, 1989). VZV infection of T-cells appears to represent a critical phase of its life cycle, providing a mechanism for viral transport from sites of initial infection to the skin. Sensory nerve axons terminate in the dermis and may be infected with VZV, allowing for retrograde transport to sensory ganglia, as it spreads through the skin layers (Annunziato *et al.*, 2000). VZV may also reach neurons by hematogenous spread. The outcome of VZV infection in these various cell types, which are differentiated and non-dividing, depends on interactions between virus proteins and host factors at the cellular level and is modulated by the innate and adaptive immune responses of the infected host (Chapter 39). VZV infection of dendritic cells is described in Chapter 39, and investigations of latency and reactivation in the human host and in rodent models are reviewed in Chapter 38. The goals of this chapter are to discuss VZV infection of T-cells and skin, which are essential target cells of the virus during primary infection, and the disease consequences of varicella in healthy and immunocompromised individuals.

Systems for evaluating determinants of VZV pathogenesis in human skin and T-cells

Immunodeficient (*scid/scid*) mice with thymus/liver (T-cell) or skin xenografts have provided a useful experimental model for examining VZV pathogenesis in vivo, known as the SCID-hu model of VZV infection (Moffat *et al.*, 1995). In this model, VZV-infected fibroblasts are injected into human tissue xenografts, which are then removed at intervals up to 21–28 days after infection. Initial experiments with T-cell xenografts showed that VZV proteins were expressed in CD4+, CD8+ and CD4/CD8+ T-cells and VZV was cultured from each T-cell subpopulation. T-cells released infectious VZV, which was an important

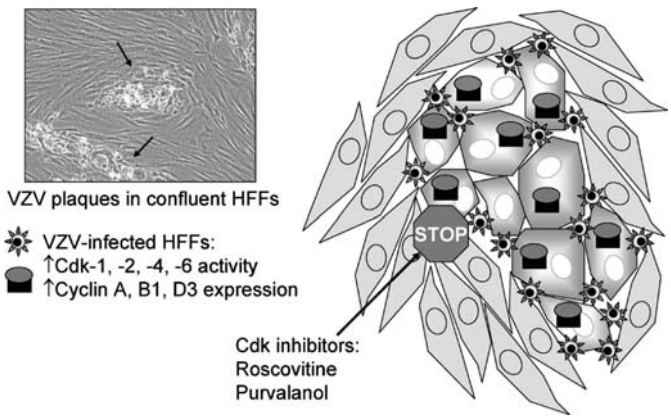

Fig. 37.1. Activation of host cyclin-dependent kinases in VZV-infected fibroblasts. VZV infects quiescent cells such as dermal fibroblasts (HFFs) to cause skin lesions. The photo of VZV plaques in HFFs (arrows) shows that the infected cells are rounded and have not fused into syncytia, though they are adherent (magnification, 10X). The uninfected cells are confluent and contact inhibited. Since they are not dividing, HFFs express basal levels of cyclin D3, and the cyclin-dependent kinases (Cdks) are inactive. Upon infection with VZV, unscheduled cyclin expression results in high levels of cyclins A, B1, and D3, which does not reflect a normal phase of the cell cycle. In association with these cyclins, several cdks are activated in VZV-infected HFFs: Cdk-1, −2, −4, and −6 (depicted as oval/rectangle heterodimers). It is not known how VZV infection dysregulates cdks or how this favors virus replication. However, when cdk activity is inhibited with roscovitine or purvalanol, VZV spread is reversibly blocked, and viral DNA synthesis is shut off. (Adapted from (Moffat *et al.*, 2004b), photo provided by Stacey Leisenfelder.)

observation because VZV replication in vitro is highly cell-associated. VZV-infected skin implants exhibited epidermal lesions that were indistinguishable from the characteristic lesions of varicella. Experiments with VZV mutants in the SCIDhu model indicate that VZV is challenged to employ more gene functions to replicate successfully in skin and T-cells in vivo, because the cellular environment in intact tissues is fundamentally different from that in cultured cells. Host cell factors such as innate immunity, cellular kinase activation, cellular transactivating proteins and protein trafficking pathways, are likely to affect virulence such that the full range of VZV gene products is needed to modify the tissue environment for effective viral pathogenesis.

Information about the molecular pathogenesis of VZV has been obtained by generating VZV recombinants with selected mutations in the viral genome using cosmid systems. Sets of four or five cosmids have been derived using genomic DNA from the parent Oka strain, pOka, a clinical isolate from Japan from which the live, attenuated varicella vaccine virus, vOka, was made (Niizuma *et al.*, 2003) and from the vOka strains used by Merck & Co., Inc. (Kemble *et al.*, 2000) and the strain deposited at the American Type Culture Collection (Cohen and Seidel, 1993). To obtain mutant viruses, alterations are made in selected VZV open reading frames (ORFs) and introduced into the cosmid that carries the gene of interest. The altered cosmid and the three or four other cosmids are transfected or transduced together into human melanoma cells. Homologous recombination between overlapping sequences generates an intact VZV genome, and if the mutation is not lethal, the virus propagates by cell-cell spread and plaques appear in the transfected cells. To discern phenotypes beyond lethality, mutant viruses are compared to isogenic parent viruses and a repaired strain, also made from cosmids. These "matched sets" of mutant, parent, and repaired viruses are evaluated in vitro using primary cell cultures and in the SCIDhu model in vivo.

Effects of VZV replication on cellular cyclin-dependent kinases and cyclins

The primary reason why VZV pathogenesis must be studied in vivo using the SCIDhu model is that the intracellular environment in human skin and thymus tissues differs greatly from the conditions in cultured cells. VZV is commonly cultivated in human melanoma cells (MeWo) or in subconfluent human fibroblasts (HFF, HELF, MRC-5, WI-38) that contain abundant metabolic precursors and enzymes involved in cell growth and division. In contrast, dermal fibroblasts, differentiated keratinocytes, and single-positive CD4+ or CD8+ T cells in xenografts in SCIDhu mice are not dividing. In these quiescent cells, regulatory proteins such as Rb and p27 suppress biosynthetic pathways for DNA replication and cell division by inhibiting transcription of cyclins and the activity of cyclin-dependent kinases (CDKs) (Olashaw and Pledger, 2002). VZV infection subverts these suppression mechanisms in an unknown manner and causes unscheduled cyclin expression and dysregulation of cyclin-dependent kinases (Fig. 37.1). In VZV-infected, confluent HFFs, high levels of CDK activity are associated with simultaneous expression of cyclins A, B1, and D3 (Leisenfelder and Moffat, 2006). This unusual protein profile is likely induced by VZV since it does not correspond with CDK and cyclin expression patterns found in cellular G0/1, S, G2, or M phases.

VZV alters the intracellular environment of resting cells by inducing kinase activity, an effect that appears to be

an essential step in virus replication because compounds that inhibit CDK activity prevent VZV spread. Roscovitine and purvalanol A are specific inhibitors of CDKs 1, 2, 5, 7, and 9 that have potent antiviral effects on VZV and other viruses (Moffat *et al.*, 2004b; Taylor *et al.*, 2004). Interestingly, as little as 5μM roscovitine or 2μM purvalanol is needed to prevent VZV replication and yet 10-fold more is needed to cause cell cycle arrest in MeWo cells. Thus VZV is acutely sensitive to levels of CDK activity, which the virus may utilize for initiation of transcription from viral promoters (CDK7 and CDK9), phosphorylation of the C-terminus of glycoprotein I (CDK1) (Ye *et al.*, 1999), and for numerous potential viral and cellular protein targets. An important role for kinase activity associated with cyclin B, presumably CDK1, is phosphorylation of IE62 since these proteins interact in the cytoplasm. Recognition sites for CDK1 are plentiful in IE62, and point mutagenesis confirmed that several are targeted by the kinase in vitro (Leisenfelder and Moffat, unpublished observations).

Investigation of events in the pathogenesis of primary VZV infection in the SCIDhu model

The clinical experience documents that primary VZV infection is initiated by inoculation of the respiratory mucosal epithelium and that the varicella rash appears after a 10–21 day incubation period (Arvin, 2001a; Cohen and Straus, 2001). Given the extreme host-range restriction of VZV, concepts about the pathogenesis of primary VZV infection have been derived from the sequence of events during primary mousepox infection (Grose, 1981). Based on this model, VZV has been thought to reach mononuclear cells in regional lymph nodes, causing a primary viremia that transports the virus to reticuloendothelial organs, such as the liver, for a phase of viral amplification. The theory has been that this amplification stage is followed by a secondary viremia in the late incubation period that carries VZV to skin sites. Recently, work using the SCIDhu model has provided experimental evidence to refine hypotheses about primary VZV pathogenesis (Ku *et al.*, 2004). According to our new model of VZV pathogenesis, VZV tropism for T-cells may facilitate viral transfer to skin (Fig. 37.2). Previous investigations in cultured cells demonstrated that T cells could be infected with VZV (Koropchak *et al.*, 1989; Soong *et al.*, 2000) and that tonsil T-cells, especially activated, memory subpopulations, are highly permissive for VZV infection (Ku *et al.*, 2002). In addition, VZV preferentially infected the activated, memory CD4+ T-cells that constitute >20% of tonsil T-cells. T-cell sub-populations that expressed the skin homing markers, cutaneous leukocyte antigen (CLA), and chemokine receptor CCR4, were also more likely to be infected and VZV did not disrupt the important chemotaxis functions of these cells. In order to investigate the hypothesis that VZV could be transferred to skin by tonsil T-cells, VZV-infected human tonsil T-cells were adoptively transferred to SCIDhu mice via intravenous injection (Ku *et al.*, 2004). The microcirculation within these skin xenografts is formed by human $CD31^+$ endothelial cells, which permits interaction with human T-cells. CD3+ T cells were detected within the epidermis, dermis and around the hair follicles in skin tissues within 24 h after injection into the mouse circulation. T-cells expressing the memory marker, CD45RO, were the predominant population. When skin xenografts were harvested at intervals after inoculation of infected T-cells, characteristic cutaneous VZV lesions were formed and progressed in size and production of infectious virus over 10–21 days. Intravenous injection of VZV-infected fibroblasts did not result in VZV transfer into skin xenografts. T-cell transfer of the virus resulted in lesions expressing the VZV proteins needed for lytic infection, such as the major immediate early ORF62 (IE62) transactivator, the ORF47 kinase and glycoprotein E (gE). VZV infection of skin resulted in extensive formation of multi-nucleated polykaryocytes, a gradual thickening of the epidermis, epidermal cell proliferation, destruction of basement membranes, and cellular degeneration. Foci of VZV-infected cells eventually extended up to surface keratinocytes but only after 14–21 days. Infectious VZV was produced throughout this period of progressive cutaneous lesion formation. Thus, the time required for VZV lesions to penetrate through the keratinocyte layer at the skin surface implies that VZV must reach cutaneous sites of replication at an early, rather than a late stage of the incubation period.

VZV alters the intracellular environment and produces lytic infection within 2 days in cultured cells in vitro. In contrast, VZV infection in skin xenografts evolved much more slowly. These observations suggested that innate immune mechanisms within the intact cutaneous tissue microenvironment in vivo might modulate VZV replication. Analyses of skin xenografts showed that interferon-α (IFN-α) and interleukin 1-α (IL-1α) were expressed constitutively in the cytoplasm of epidermal cells in uninfected skin (Ku *et al.*, 2002). After VZV infection, IL-1α was translocated to the nuclei of cells expressing VZV proteins, but remained in the cytoplasm of adjacent, uninfected cells. TNF-α expression was not present in uninfected skin and was not induced by VZV infection. Importantly, interferon-α (IFN-α) was not expressed in VZV-infected cells but was up-regulated in neighboring uninfected epidermal cells within the skin

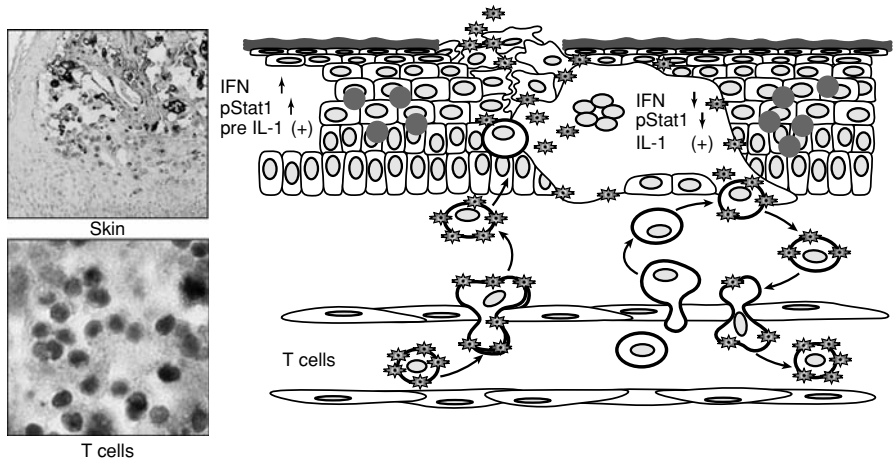

Fig. 37.2. A model of the pathogenesis of primary VZV infection. This figure illustrates new concepts about VZV pathogenesis and immunobiology that have emerged from experiments in the SCIDhu mouse model. The upper left panel shows the appearance of a mature VZV lesion in a SCIDhu skin xenograft, stained for VZV protein expression with a polyclonal human anti-VZV IgG antiserum. The lower left panel shows VZV infection of T-cells in a SCIDhu T-cell xenograft, detected by *in situ* hybridization with a VZV DNA probe. The right panel is a diagram depicting proposed events in the pathogenesis of VZV infection of skin. According to this model, T-cells within the tonsil lymphoid tissues become infected by VZV transfer into these migratory cells of the immune system following the initial inoculation of respiratory epithelial cells with the virus. Infected T-cells enter the circulation and transport the virus to the skin shortly thereafter, exiting through capillary endothelium by the usual mechanisms for trafficking of migratory T-cells. The infected T-cells then release infectious VZV at skin sites of replication. The remainder of the 10–21-day incubation period is the interval required for VZV to overcome the innate IFN-α response in enough epidermal cells to create the typical vesicular lesions containing VZV at the skin surface, as shown in the upper left panel. Signaling of enhanced IFN-α production in adjacent skin cells prevents a rapid, uncontrolled cell-cell spread of VZV. Additional crops of varicella lesions may result when T-cells traffic through early stage cutaneous lesions, become infected and produce a secondary viremia. This process continues until host immune responses trigger the up-regulation of adhesion molecules and mediates the clearance of the virus by VZV-specific antiviral T-cells. (Adapted from Ku *et al.*, 2004.)

xenograft. The phosphorylation state of Stat1 protein is a marker for activation or suppression of the IFN-α pathway because IFN-α binding to its receptors induces Stat1 phosphorylation by JAK kinases. Without phosphorylation, Stat1 is not translocated to cell nuclei and production of IFN-α does not occur. In VZV-infected skin, phosphorylated Stat1 was localized to nuclei in neighboring uninfected epidermal cells, but it was not detected in cells expressing VZV proteins. In uninfected skin, Stat1 was not phosphorylated and Stat1 and IFN-α remained cytoplasmic. Another mediator of the innate immune response, the transcription factor NF-κB, also remained in the cytoplasm of VZV-infected skin cells in SCIDhu implants (Jones and Arvin, 2006). These experiments indicated that VZV replication was associated with expression of a gene product(s) that inhibited antiviral IFN-α production in foci of infected skin cells in vivo by interference with Stat1 activation. To further document the role of the IFN-α response in regulating VZV infection and cell-cell spread between epidermal cells within intact skin tissue in vivo, skin xenografts were inoculated with VZV and SCIDhu mice were given neutralizing antibody against the human IFN-α/β receptor, in order to block type I IFN activity. In these experiments, infectious VZV titers were ten-fold higher in skin specimens when IFN signaling was inhibited by receptor blocking and the cutaneous lesions were substantially larger.

Because SCID mice lack the capacity to develop an adaptive, antigen-specific immune response, the progression of VZV infection in skin xenografts is controlled only by innate immunity. In the intact human host, biopsies of varicella skin lesions show a local inflammatory response surrounding the infected cells. The migration of immune cells into damaged tissues is signaled by the up-regulation of adhesion molecules on the vascular endothelial cells. Comparative analyses of infected skin xenografts and human VZV lesion biopsies permitted an examination of whether these changes could be induced by viral replication *per se*. Cutaneous lesions in patients biopsied at the onset of the varicella rash showed extensive expression of E-selectin, ICAM-1, and VCAM-1, whereas these proteins were not detected in capillaries of infected skin xenografts. Many infiltrating mononuclear cells were detected, most of

which expressed CD4 or CD8, and included predominantly $CD45RO^+$ memory T-cells and skin homing CLA^+ and $CCR4^+$ T-cells. In contrast, adoptive transfer of PBMC to SCIDhu mice showed no enrichment for these effector T-cell populations in VZV infected skin. These differences in adhesion molecule expression and in mononuclear cell profiles in VZV lesion biopsies and in VZV-infected skin xenografts suggested that recruitment and/or retention of inflammatory T-cells required signals provided by host cellular immunity.

Considered together, data showing VZV infection of T cells in vitro (Ku *et al.*, 2002; Soong *et al.*, 2000) and these experiments in the SCIDhu model suggest that T-cell tropism plays an essential role in VZV pathogenesis. The prolonged varicella incubation period appears to represent the time required for VZV to overcome previously unrecognized, but potent innate antiviral responses, especially IFN-α production, mediated directly by epidermal cells in vivo. The initial phase of VZV pathogenesis is also likely to be facilitated by the failure of VZV to trigger up-regulation of inflammatory adhesion molecules on capillary endothelial cells in skin and virus-mediated modulation of MHC I and MHC II expression (see elsewhere in this volume).

The role of VZV glycoproteins in T-cell and skin tropism

As is true of all herpesviruses, VZV glycoproteins have multiple functions that affect tissue tropism both within cells during virus assembly, and outside cells when they are expressed on plasma membranes and virion envelopes. VZV encodes glycoproteins designated gB, gC, gE, gH, gI, gK, gL, gM and gN. However, characterization of the functions of most of these proteins is limited. VZV is unique among the alphaherpesviruses in having no gD homologue. In transfected and VZV-infected cells, glycoproteins form gE/gI and gH/gL heterodimers and gE homodimers; noncovalent interactions between gB/gE and gH/gE have also been identified (Cole and Grose, 2003). Recycling of glycoproteins from the cell surface to the trans-Golgi network (TGN) is regulated to balance envelopment in the TGN, where infectious virions are formed, with the cell fusion that spreads VZV genomes to neighboring cells even when formation of intact virion particles is limited. The VZV glycoproteins, gB, gC, gE, gH, gI, gK, and gL have been shown to be or are likely to be structural components of the virus (Cohen and Straus, 2001; Mo *et al.*, 1999, 2002). In addition to studies of the glycoproteins using expression systems, deletions or targeted mutations of these genes that are not lethal have yielded recombinant VZV mutants for investigation of glycoprotein function in cultured cells and in SCIDhu T cell and skin xenografts in vivo. VZV gB, gE and gK have been demonstrated to be essential for VZV replication in cultured cells, based on failure to generate infectious virus from cosmids with deletions of these genes, and the rescue of infectivity when the deletion is complemented by insertion of the gene into a non-native site and in cell lines. In the SCIDhu model, interactions between VZV glycoproteins at internal sites of virion assembly and surface membranes determine the ability of VZV to replicate in differentiated human T-cells and skin. This summary focuses particularly on evaluations of the contributions of VZV glycoproteins to viral pathogenesis in T-cell and skin xenografts in vivo.

Glycoprotein C

VZV gC is the product of ORF14 (Davison and Scott, 1986). The role of gC in infectivity for human skin was assessed using gC negative mutants of vOka and VZV-Ellen (Moffat *et al.*, 1998). Whereas all of these VZV strains replicated well in tissue culture, only low passage clinical isolates were fully virulent in skin, as shown by infectious virus yields and analysis of xenografts for VZV DNA and viral protein synthesis. All strains except the gC-null Ellen strain retained some capacity to replicate in human skin, but cell-free virus was recovered only from xenografts infected with pOka or VZV-S. An HSV-1 mutant lacking gC expression was also deficient in skin infectivity. These SCID-hu mouse experiments show that gC, which is dispensable for replication in tissue culture, plays a critical role in the virulence of the human alphaherpesviruses, VZV and HSV-1, for human skin.

Glycoprotein E

Whereas the homologous protein in the other alphaherpesviruses is dispensable in cultured cells, VZV gE, encoded by ORF68, is essential for replication (Ku *et al.*, 2002). The functions of gE were analyzed further by creating point mutations or deleting the short 62 amino acid C-terminal domain (Moffat *et al.*, 2004a). These mutants were designed based on observations about functional motifs made using gE expression systems (Cole and Grose, 2003). Mutations were introduced in YAGL (aa582–585), which mediates gE endocytosis, AYRV (aa568–571), the motif that targets gE to the trans-Golgi network (TGN), and SSTT, which is an "acid cluster" comprising a phosphorylation motif (aa588–601). A substitution Y582G in YAGL prevented gE

endocytosis, and the Y569A mutation interfered with gE shuttling from the Golgi to the TGN in reports using transient expression methods. These changes were introduced into the viral genome using VZV cosmids and residues S593, S595, T596, and T598 were changed to alanines to alter phosphorylation. These experiments demonstrated a hierarchy in the contributions of gE C-terminal motifs to VZV replication in vitro and to virulence in the SCIDhu model. Deleting the gE C-terminus or mutating the YAGL motif were lethal for VZV replication in vitro. Mutations of AYRV and SSTT were compatible with recovery of VZV, but the AYRV mutation resulted in decreased plaque size and virus production in vitro. When the rOka-gE-AYRV and rOka-gE-SSTT mutants were evaluated in skin and T cell xenografts in SCIDhu mice, interference with TGN targeting was associated with substantial attenuation, especially in skin, whereas the SSTT mutation did not alter VZV infectivity in vivo. Thus, the gE C-terminus contains domains that are essential for VZV replication or are determinants of VZV virulence in differentiated dermal and epidermal cells and T-cells within intact tissue microenvironments in vivo. In addition to C-terminal functions, VZV gE has a unique N-terminal region from amino acids 1–188 (Berarducci *et al.*, 2006). Mutagenesis of this gE ectodomain region identified subdomains essential for replication, cell-cell spread and secondary envelopment and for VZV skin tropism.

Whereas VZV has been considered to be highly antigenically stable, VZV-MSP is a recently discovered wild type virus that has lost an immunodominant B-cell epitope in the gE ectodomain (Mo *et al.*, 2002). This gE "escape mutant" virus exhibited an unusual pattern of egress. When VZV-MSP was evaluated in SCIDhu skin xenografts, the spread of the VZV-MSP variant was accelerated significantly. The cytopathologic changes produced after 21 days by isolates that had the prototypical gE sequence were demonstrated at 14 days in skin xenografts infected with VZV-MSP. Thus, VZV-MSP is a naturally occurring variant with a gE mutation that is associated with a phenotype of enhanced cell–cell spread in vitro and in vivo.

Glycoprotein I

VZV mutants with deletions of gI, encoded by ORF67, can replicate in melanoma cells and fibroblasts, although not in Vero cells (Cohen and Nguyen, 1997; Mallory *et al.*, 1997). Since gI was dispensable in cell culture, gI deletion mutants were evaluated for their capacity to infect human cells in SCIDhu xenografts. Deleting gI was lethal for VZV replication in differentiated skin and T cells in vivo (Moffat *et al.*, 2002). Restoring gI into the mutated VZV genome was associated with the recovery of VZV virulence. Thus, gI is essential for VZV pathogenesis.

Analyses of the gI promoter in expression systems has demonstrated that it contains an activating upstream sequence (AUS) that binds cellular transcription factors Sp1 and USF (Specificity factor 1, Upstream Stimulatory Factor), and the viral transactivator ORF29 DNA binding protein which mediates enhancement of immediate early 62 (IE62)-induced transcription (He *et al.*, 2001). This information was used to design mutants from VZV cosmids in order to evaluate the contributions of these motifs to VZV replication in vitro and in vivo (Ito *et al.*, 2003). Recombinants rOkagI-Sp1 and rOkagI-USF, with two substitutions in Sp1 or USF sites, replicated like rOka in vitro, but infectivity of rOkagI-Sp1 was significantly impaired in skin and T cells in vivo. A double mutant, rOKAgI-Sp1/USF, did not replicate in skin, but yielded low titers of infectious virus in T-cells. The repair, rOkagI:rep-Sp1/USF, was as infectious as rOka. Thus, disrupting gI promoter sites for cellular transactivators altered VZV virulence in vivo, with variable consequences related to the cellular factor and the host cell type. Mutations in the ORF29 responsive element of the gI promoter were made by substituting each of four 10 base pair blocks in this region with a 10 base pair sequence, GATAACTACA, that was predicted to interfere with enhancer effects of the ORF29 protein. One of these mutants, designated rOKAgI-29RE-3, had diminished replication in skin and T-cells, indicating that ORF29 protein-mediated enhancement of gI expression contributes to VZV virulence. These experiments demonstrated that VZV pathogenesis is influenced by interactions of cellular transactivators with the gI promoter. Significantly, comparisons of the effects of the gI promoter mutants on growth in skin and T-cells indicated that cellular transactivators can have consequences for virulence that are cell-type specific. Mutations within promoters of viral genes that are non-essential in vitro should allow construction of recombinant herpesviruses that have altered virulence in specific host cells in vivo, and may be useful for designing herpesvirus gene therapy vectors and attenuated vaccines.

The role of regulatory proteins and viral kinases in T-cell and skin tropism

Although the difficulty of generating sufficient quantities of infectious cell-free virus has prevented experimental analysis, VZV replication is presumed to occur through a sequential expression of immediate early, early and late genes (Chapter 10). VZV regulatory proteins include viral

transactivating proteins and viral kinases, which may also be structural components of the virion tegument. While the task is far from complete, some of these genes have been analyzed for their contributions to VZV replication in cultured cells and in the SCIDhu model of VZV pathogenesis in vivo.

IE62 protein

The IE62 protein is the major VZV transactivating protein, required for expression of all viral genes tested to date (Kinchington *et al.*, 1992, 1994). It is encoded by the duplicated genes, ORF62 and ORF71. Experiments in which pOka cosmids were mutated to delete ORF62, ORF71, or the ORF62/71 gene pair demonstrated that at least one copy of ORF62 was required for VZV replication, as expected (Sato *et al.*, 2003a). Restoring a single copy of ORF62 into a non-native site in the U_S region of the VZV genome resulted in some, albeit reduced, VZV replication in vitro. VZV replication persisted despite introducing targeted mutations in IE62 binding sites that mediate interaction with the IE4 protein. Related experiments demonstrated that the ORF4 gene is essential in VZV (Sato *et al.*, 2003b). Interestingly, when a single copy of ORF62 or ORF71 was deleted, recombination events during cosmid transfection repaired the defective repeat region in some progeny viruses. Mixtures of single copy rOkaΔ62 or rOkaΔ71 and repaired rOka generated by recombination of the single copy deletion mutants was detected in some skin xenografts infected with these recombinants. The diminished replication of the pOka mutants with a single copy of ORF62 at the non-native site was associated with a complete block in VZV infection of skin xenografts in vivo. Although insertion of ORF62 into the non-native site permitted replication in cell culture, ORF62 expression from its native site was necessary for cell-cell spread in differentiated human skin tissues in vivo.

IE63 protein

The IE63 protein is encoded by ORF63 and is duplicated in the VZV genome as ORF70. IE63 protein is a nuclear phosphoprotein with some homology to HSV-1 ICP22. Sequence analysis indicates that IE63 is related to HSV-1 $U_S1.5$ protein, which is expressed colinearly with ICP22 (U_S1) (Baiker *et al.*, 2004). Removing one copy of the duplicated gene, either ORF63 or ORF70, was compatible with VZV replication in vitro (Sommer *et al.*, 2001). VZV was not recovered from transfections done with a dual deletion cosmid, but infectious virus was generated when ORF63 was cloned into the non-native site in the Us region. IE63 protein interacts directly with ORF62, the major immediate early transactivating protein of VZV. The importance of IE62 protein for VZV replication is suggested by the observation that ORF63/ORF70 could be removed and yield infectious virus in vitro if deleted cosmids were transfected along with a plasmid expressing IE62 (Cohen *et al.*, 2004).

The potential functional domains of IE63 protein were analyzed by creating 22 ORF63 mutations in expression plasmids and in the VZV genome. The effects of IE63 phosphorylation and nuclear localization, and IE63 binding to IE62, were evaluated by transient transfection and by replication of the mutant viruses. Briefly, IE63 aa55–67 constituted the IE62 binding site, with R59/L60 being critical residues; S165, S173 and S185 in the IE63 center region were phosphorylated by cellular kinases; and mutations in two putative nuclear localization signal (NLS) sequences changed intracellular IE63 distribution from a nuclear to a cytoplasmic/nuclear pattern. Infectious VZV was recovered with three of the 22 mutations in ORF63. Each of these three IE63 mutants had a single alanine substitution (T171A, S181A or S185A). The IE63 mutants, rOka/ORF63rev[T171], rOka/ORF63rev[S181] and rOka/ORF63rev[S185], replicated less efficiently, had a small plaque phenotype in vitro and had less production of gE and ORF47, indicating that IE63 was involved in expression of these early and late gene products. Virulence of the three IE63 mutants was reduced markedly in skin xenografts, but infection of T-cell xenografts was not affected. The fact that these IE63 mutants were attenuated in skin but not T-cells, suggests that the contribution of the IE63 tegument/regulatory protein to VZV pathogenesis differs depending on the human cell types and tissues that are targeted for infection.

ORF64 protein

ORF64 is duplicated as ORF69 and it has some sequence homology to the HSV-1 Us10 gene, which exists as a single copy. When ORF64 and ORF69 were deleted, either separately or together, one copy at either location in the genome was sufficient to yield infectious virus with growth kinetics and plaque morphology indistinguishable from the parent virus (Sommer *et al.*, 2001). Removing both ORF64 and ORF69 caused an abnormal plaque phenotype made up of very large multinucleated syncytia. Single and dual ORF64/ORF69 mutants were as infectious as the parent and repaired viruses when evaluated in human T-cells in vitro and in human skin xenografts in the SCIDhu mouse model of VZV pathogenesis.

ORF10 Protein

ORF10 encodes a tegument protein that enhances transactivation of VZV genes. Analysis of pOkaΔ10 and ORF10 point mutants with disruption of the acidic activation domain and the putative motif for binding human cellular factor-1 (HCF-1) showed no effects on replication, IE gene transcription or virion assembly in vitro (Che *et al.*, 2006). However, epidermal cells in SCIDhu skin xenografts infected with pOkaΔ10 had significantly fewer DNA-containing nucleocapsids and complete virions; extensive aggregates of intracytoplasmic viral particles were also observed. Altering the activation or the putative HCF-1 domains of ORF10 protein had no consequences for VZV skin infection. Deleting ORF10 did not impair VZV T-cell tropism in vivo. Thus, ORF10 protein is necessary for efficient VZ virion assembly and is a VZV virulence determinant in epidermal and dermal cells in vivo.

ORF47 protein

ORF47 encodes a serine/threonine protein kinase that is in a class of conserved herpesvirus proteins that are homologous to HSV-1 U_L13. ORF47 protein also has similarities to the casein kinase II family of cellular proteins (Cole and Grose, 2003). ORF47 appears to be a component of the virion tegument. VZV mutants that did not express ORF47 protein were made by inserting stop codons into the gene, producing ROka47S, which was shown to replicate as well as intact ROka in an infectious focus assay (Heineman *et al.*, 1996). However, these findings were not predictive of the consequences of blocking ORF47 protein synthesis in vivo, since ORF47 protein was essential for VZV infection of human T cells and skin (Moffat *et al.*, 1998). Restoring ORF47 into the genome of the ROKA47S mutant reconstituted the T cell and skin tropism of the virus. Thus, ORF47 protein functions are necessary in differentiated cells that are involved in VZV pathogenesis in vivo.

In order to further investigate the role of the ORF47 protein, VZV mutants were made that expressed a truncated ORF47 protein, by deleting the C-terminus, and that had mutations that disrupted conserved putative kinase motifs in ORF47 protein (Besser *et al.*, 2003). The mutants were tested for replication, phosphorylation and protein-protein interactions in vitro and allowed an assessment of the effects of specifically eliminating the kinase activity of ORF47 protein on VZV replication in vivo. The ORF47 C-terminal truncation mutants (rOka47ΔC) and those that disrupted the DYS kinase motif (rOka47D-N) had no ORF47 kinase activity. However, binding to IE62 protein was mapped to the N-terminal domain and was preserved. Cells infected with these ORF47 kinase defective mutants exhibited marked nuclear retention of ORF47 and IE62 proteins in vitro. Even though virus titers were not altered based on an infectious focus assay, the electron microscopy analysis of cultured cells infected with the kinase defective mutants showed severely impaired virion assembly and transport of virions to cell surfaces. Normal VZV virion assembly appears to require ORF47 kinase function. Nevertheless, rOka47ΔC or rOka47D-N-infected cells showed VZV-induced cell fusion and syncytia formation.

With regard to pathogenesis, ORF47 protein mutations that eliminated the ORF47 kinase function caused substantial reductions in the capacity to replicate and produce cutaneous lesions in skin xenografts in the SCIDhu model. However, in contrast to the complete ORF47 null mutant, rOKA47S, some replication occurred in skin in vivo if the capacity of ORF47 protein to bind IE62 protein was intact, as shown in experiments with rOka47ΔC and rOka47D-N. ORF47 kinase activity was important for VZV infection and cell-cell spread in human skin in vivo, but preserving the capacity of ORF47 protein to form complexes with IE62 protein, both of which are VZV tegument components, appeared to be the *sine qua non* for VZV infection of skin in vivo. In contrast to the skin experiments, when the kinase defective rOka47ΔC and rOka47D-N mutants were evaluated in T-cell xenografts, no infectious virus was made in vivo (Besser *et al.*, 2004). These observations were similar to the data obtained in T-cell xenografts infected with ROka47S, when no ORF47 protein was made. The comparison of the growth of kinase-defective ORF47 mutants in skin vs. T-cells suggested the hypothesis that fundamental requirements for VZV pathogenesis in skin and T-cells differ in vivo. Even though virion assembly was much diminished and intracellular trafficking of ORF47 and IE62 proteins, both components of the tegument, and of gE, was aberrant in skin in the absence of ORF47 kinase activity, VZV polykaryocytes were generated by rOka47ΔC and rOka47D-N. Thus, some cell fusion was induced by ORF47 mutants in skin and cell–cell spread occurred even though virion formation was deficient. In contrast, impaired virion assembly by ORF47 mutants was associated with a complete elimination of the capacity to infect T-cells in vivo. Since VZV-infected T-cells do not undergo cell fusion even when most cells in the T cell xenograft have been infected, transfer of incomplete virions by cell–cell fusion does not occur. Instead, virus appears to be released from T-cells for entry into uninfected T-cells in other regions of the xenograft. Considered together, these observations make it plausible to suggest that formation of complete virions

and their release is essential for VZV T-cell tropism, creating a differential requirement for virion assembly during the pathogenesis of VZV infection of T-cells and skin.

ORF66 protein

ORF66 encodes a second serine/threonine protein kinase homologous to HSV U_S 3. Like the ORF47 protein kinase, ORF66 protein was shown to be dispensable for VZV replication in cultured cells by creating ROka66S stop codon mutants. Again, ROka66 mutants replicated as well as intact ROka in cultured cells. Eliminating ORF66 expression did not impair replication in SCIDhu skin xenografts, as compared to the vaccine-derived ROka parent (Moffat *et al.*, 1998). In contrast, ORF66 defective VZV mutants had a significant decrease in their capacity to replicate in T-cell xenografts in vivo. Thus, ORF66 protein appears to be a viral kinase that is necessary to VZV T-cell tropism. When ORF66 expression was blocked in pOka, growth and VZ virion formation was reduced in T-cells in vivo, infected T-cells were more susceptible to apoptosis and pOka66S mutants had less capacity to interfere with induction of the interferon (IFN) signaling pathway (Schaap *et al.*, 2005). Thus, ORF66 kinase appears to have a unique role during T-cell infection and supports VZV T cell tropism by contributing to immune evasion and enhancing survival of infected T-cells.

Disease consequences of primary VZV infection in healthy and immunocompromised hosts

The clinical pattern of primary VZV infection is highly predictable, beginning with an incubation period of 10–21 days following a close exposure of a susceptible individual to another person with varicella or in some cases, herpes zoster (Arvin, 2001b). In contrast to other herpesviruses, primary VZV infection almost always causes symptoms although the diagnosis is missed when the child has only a few lesions and no identified exposure. Varicella often begins with a prodrome of fever, malaise, headache and abdominal pain. These initial symptoms last about 24–48 hours before skin vesicles are noted and are more common in older children and adults. The occurrence of a cell-associated VZV viremia has been well documented during the last few days of the incubation period and for a few days after the cutaneous rash appears, when specimens are tested by tissue culture or for VZV DNA (Asano *et al.*, 1990; Gershon *et al.*, 1978; Koropchak *et al.*, 1989, 1991; Ozaki *et al.*, 1986). Viral cultures of PBMC demonstrate that infectious virus can be recovered from PBMC; VZV was isolated from 11%–24% of PBMC samples taken from healthy individuals with varicella less than 24 h after the rash had appeared. DNA methods are more sensitive, with VZV being detected in 67%–74% of samples tested by in situ hybridization or PCR. Although viral cultures do not yield infectious virus, PCR methods indicate that VZV is present in oropharyngeal specimens just before and after the appearance of skin lesions. The estimated frequency of VZV infection of PBMC from healthy individuals with varicella was approximately 0.01%–0.001%, as detected by in situ hybridization (Koropchak *et al.*, 1989). According to our proposed model of the pathogenesis of primary VZV, this viremic phase may represent the infection of T cells migrating through infected skin sites and re-entering the circulation (Fig. 37.1). This early phase of the illness is usually associated with systemic symptoms, including fever and fatigue, presumably related to cytokine responses; varicella-related fever is usually mild (less than 101.5 °F). The cell-associated VZV viremia is transient, usually resolving within 24–72 hours after the onset of the rash in healthy children and adults. Primary VZV infection is often accompanied by a reduction in the numbers of circulating lymphocytes but this finding is probably secondary, rather than being due to cell destruction by the virus. Mild upper respiratory symptoms and diarrhea may occur but severe respiratory or gastrointestinal illness is rare.

The lesions caused by VZV replication in the skin appear first as small erythematous papules, each of which then evolves within about 12–24 hours to surface vesicles that are filled with clear fluid and surrounded by erythema – the so-called "dew drop on a rose petal." The first skin lesions in patients with varicella often appear on the face and scalp, or on the chest or back and are pruritic. Formation of multinucleated epithelial cells with intranuclear eosinophilic inclusions and vasculitis involving small blood vessels occurs during the early maculopapular stage. VZV virions are detected in keratinocytes and also in capillary endothelial cells by electron microscopy. VZV is delivered to mucous membrane sites as well as to skin, where it produces ulcers in the oropharynx, conjunctivae and vagina. Vesicles result from a progressive ballooning degeneration of epithelial cells and coalescence of fluid-filled vacuoles between cells. The numbers of VZV-infected cells at the base of the lesion increases during this phase and cell-free virus is released into vesicular fluid. Each lesion begins to become cloudy and crusted within about 48 hours and infectious virus is usually no longer detected after about 72–96 hours. Healing reflects the replacement of epithelial cells at the base of the lesion by cellular proliferation. New skin and mucous membrane lesions continue to develop for a period of 3–5 days in most children, with

a range of 1–7 days. Over the 1–7 day course of primary VZV infection, as few as 10 to more than 1500 lesions may appear; on average, healthy children have about 100–300 lesions. Older children and adults, those who are secondary household cases and patients with skin trauma, such as sunburn or eczema are more likely to have more cutaneous and mucous membrane lesions. The crops of lesions that appear later in the clinical course of varicella are usually on the arms and legs. Vesicle formation may be abortive, with little or no infectious virus being detected, presumably due to the induction of antigen-specific T cells by this point in the infection (Chapter 39). VZV lesions are usually superficial and do not leave scars except at the sites of the earliest skin replication; residual scars can often be seen along the hairline or eyebrows.

Secondary bacterial infection of skin lesions is the most common complication of primary VZV infection in healthy children. These infections are most often due to *Staphylococcus aureus* or *Streptococcus pyogenes* (group A beta-hemolytic streptococcus) (Dunkle *et al.*, 1991; Jackson *et al.*, 1992). Skin and mucosal damage may provide a portal of entry for these organisms such that bacteremia occurs and the organisms reach deep tissue sites. Thus, varicella may be associated with staphylococcal or streptococcal pneumonia, arthritis or osteomyelitis. Varicella lesions often involve the eyelids and ocular conjunctivae but serious eye complications are rare; unilateral anterior uveitis or corneal lesions may develop but long-term damage is unusual (Liesegang, 1991).

VZV has the capacity to infect the epithelial cells that line the pulmonary alveoli, and to induce edema and an extensive infiltration of mononuclear cells into the alveolar septae. The result of this process can be a severe viral pneumonia. Active VZV replication in the lungs is very unusual in healthy children with varicella. However, the increased morbidity and mortality caused by primary VZV infection in adults is accounted for by their much greater susceptibility to varicella pneumonia (Krugman *et al.*, 1957). Interstitial inflammation and the desquamation of alveolar lining cells into the alveoli has the potential to block the effective transfer of oxygen from the alveolar spaces into the pulmonary capillaries. The consequence is severe hypoxemia and respiratory failure. Most patients with varicella pneumonia develop cough and dyspnea several days after the onset of the cutaneous rash, which suggests that the virus reaches pulmonary epithelial sites during the later viremic phase. Physical abnormalities associated with varicella pneumonia may be difficult to detect because early signs are often limited to fever and tachypnea. The chest radiograph usually shows interstitial pneumonitis with diffuse bilateral infiltrates and perihilar nodular densities but may appear relatively benign even when patients have severe hypoxia. Severe varicella pneumonia may be fatal even with antiviral therapy (Chapter 65) and assisted ventilation.

Healthy children with varicella often have mild, sub clinical hepatitis, detected by minor abnormalities of liver function tests. These abnormalities may reflect an inflammatory response or some limited viral replication in the liver during primary VZV infection. Liver involvement is usually asymptomatic but children with the highest elevation of liver function tests may have severe vomiting. Extensive VZV infection of hepatocytes, with widespread hepatocellular destruction due to virus-induced cell lysis is a rare occurrence but is associated with fulminant hepatic failure.

In addition to its neurotropism for cells in the sensory ganglia, VZV can cause encephalitis and cerebellar ataxia. Meningoencephalitis and cerebellar ataxia are the major clinical signs of VZV-related damage to the central nervous system; some patients have signs of both cerebral and cerebellar disease (Johnson and Milbourn, 1970; Peters *et al.*, 1978). VZV was the cause of encephalitis in 13% of cases of defined etiology in CDC surveillance studies from 1972 and 1977. Although these syndromes are the most common neurologic complications of varicella, information about the pathogenesis of these disorders is limited because most children recover. How primary VZV infection might produce cerebellar ataxia is of interest because VZV is the most common cause of this syndrome in healthy children. VZV has been recovered from the brain tissue of immunocompromised children with fatal varicella encephalitis, suggesting that this syndrome might be caused by direct infection. However, it is speculated that these neurologic manifestations of primary VZV infection may be immune-mediated, for the most part. The symptoms are typically transient but neurologic complications are the second most frequent indication for hospitalization of otherwise healthy children with varicella. The onset of neurologic complications follows the appearance of the rash by several days but a few case reports describe encephalitis and ataxia beginning before skin lesions have appeared. The symptoms of encephalitis are sudden changes in the level of consciousness and generalized seizures; the signs may be meningeal, e.g., nucal rigidity, rather than encephalitic in some cases. The cerebellar syndrome is characterized by a gradual onset of irritability, ataxia, nystagmus and speech disturbances. The cerebrospinal fluid usually shows a mild mononuclear cell inflammatory pattern, with a predominance of lymphocytes, a somewhat elevated protein (<200 mg) and normal glucose, or in some cases, the cerebrospinal fluid may be normal (Gershon *et al.*, 1980).

Children under 5 and adults appear to be the most susceptible to central nervous system complications. Not surprisingly, the highest risk of fatal complications appears to be associated with encephalitis rather than cerebellar ataxia. Varicella encephalitis usually resolves within 24–72 hours, even without antiviral therapy. Information about the risk of long-term sequelae after varicella encephalitis is limited; whereas most recover fully, some patients have recurrent seizures and permanent neurologic deficits (Johnson and Milbourn, 1970). The signs of cerebellar ataxia can persist for days or weeks. Among the rare neurologic complications of varicella are transverse myelitis, optic neuritis and very rarely, Guillain-Barre syndrome.

Primary VZV infection can be associated with thrombocytopenia and coagulopathy, although these manifestations are unusual in healthy individuals. The signs of these complications include hemorrhage into the skin vesicles, petechiae, purpura, epistaxis, hematuria and gastrointestinal bleeding. The mechanisms by which thrombocytopenia may be induced include reduced production of platelets and decreased platelet survival; vasculitis, transient hypersplenism or intravascular coagulopathy may be involved. As described for varicella pneumonia, adults are at higher risk for acute hemorrhagic complications of varicella than children. Purpura fulminans, due to arterial thrombosis, is a very rare but life-threatening complication of varicella. Immune-mediated thrombocytopenia may also occur, with symptoms developing from 1 to 2 weeks or longer after varicella. Whether acute or later in their onset, bleeding complications may last for several weeks, but the thrombocytopenia usually resolves completely. Inflammatory damage to the kidneys, presenting as nephritis, is an unusual, late complication in children and adults with varicella; it is possible that this syndrome is due to secondary group A streptococcal infection. A few cases of nephritic syndrome and hemolytic uremic syndrome have been described in children with primary VZV infection. Viral arthritis, diagnosed by the isolation of VZV from joint fluid, is unusual and resolves spontaneously within 3–5 days and has not been associated with residual damage. Myocarditis, pericarditis, pancreatitis and orchitis are other very rare complications of primary VZV infection. The risks of varicella in healthy children and adults have been reduced substantially by the introduction of live attenuated varicella vaccines (Chapter 70).

Varicella in the immunocompromised host

Primary VZV infection was often a life-threatening illness in immunocompromised children before the introduction of acyclovir (Chapter 65) and can be attributed to the delayed or failed induction of VZV-specific cellular immunity (Chapter 39). Most information about the clinical course of varicella in high-risk patients is based on observations in children with leukemia and other childhood malignancies. These children often have prolonged fever and a much more extensive rash and continued formation of new lesions. Serious complications result from unchecked viral dissemination by cell-associated viremia to the lungs, liver and in some cases to the central nervous system. Immunocompromised children develop varicella pneumonia, hepatitis, coagulopathy and meningoencephalitis (Feldman and Lott, 1987; Myers, 1979). Whereas new varicella lesions are unusual after 3–5 days in most healthy children, new lesions may appear for >7 days and resolution of the lesions may take 14 days. Susceptibility to secondary bacterial infections is typically enhanced in children receiving chemotherapy or radiation because of the granulocytopenia induced by treatment of the malignancy. As is the case in healthy adults, most varicella-related deaths result from pneumonia that develops shortly after the appearance of the rash. Before antiviral drugs were available, varicella pneumonia progressed rapidly with most deaths occurring within a few days due to untreatable hypoxemia. Varicella pneumonia is often associated with hepatitis, which can progress to liver failure. Again, as is true in healthy adults, hemorrhage into varicella lesions is a clinical sign of life-threatening coagulopathy, due to thrombocytopenia and altered production of clotting factors. VZV dissemination can also cause meningoencephalitis. Severe abdominal or back pain is a clinical sign of serious primary VZV infection in high-risk patients but the etiology of the symptoms is unknown; it is possible that it is related to early infection of sensory ganglia by hematogenous spread of the virus. Other complications of disseminated varicella in children with malignancy include myocarditis, nephritis, pancreatitis, necrotizing splenitis, esophagitis, and enterocolitis.

Children who receive kidney, liver or other solid organ transplants may also develop progressive varicella as a result of the immunosuppressive drugs given to prevent rejection of the transplanted organ (Feldhoff *et al.*, 1981). Varicella pneumonia appears to be a less frequent complication than hepatitis and coagulopathy in kidney transplant patients. Steroid therapy for chronic diseases, including rheumatoid arthritis, nephrotic syndrome and ulcerative colitis, may lead to severe varicella. Asthma patients given high doses of prednisone, especially during the incubation period, are also at risk, but chronic low-dose steroid therapy does not usually result in varicella complications. The immunologic deficits caused by human immunodeficiency virus (HIV) infection are

associated with prolonged, recurrent varicella and with chronic, hyperkaratotic skin lesions but varicella pneumonia, hepatitis and other manifestations of dissemination are unusual compared to children with malignancies or organ transplants (Jura *et al.*, 1989; Kelley *et al.*, 1994). Any of the rare genetic disorders that interfere with the acquisition of antigen-specific T cell immunity results in very high risk of fatal varicella. These diseases include severe combined immunodeficiency disorder, adenosine deaminase deficiency, nucleoside phosphorylase deficiency and cartilage hair hypoplasia/short-limbed dwarfism; serious varicella also occurs in some children with Wiskott–Aldrich syndrome and ataxia telangiectasia.

Varicella in pregnancy and the newborn

Most adults are immune, but susceptible pregnant women appear to be predisposed to severe varicella at rates higher than the enhanced risk associated with primary VZV infection in all healthy adults. Varicella pneumonia is the predominant complication and appears to be more common with varicella acquired in late gestation (Pastuszak *et al.*, 1994). From the limited information available, the risk of fatal varicella, due to pneumonia, appears to be ~1%–2% (Enders *et al.*, 1994). When primary VZV infection occurs in early pregnancy, the virus can be transferred across the placenta to the developing fetus. The frequency of viral transfer is higher than the risk of fetal damage, as shown by postnatal testing of infants for VZV-specific immunity and the occurrence of zoster in early childhood among infants with no symptoms of intrauterine VZV infection (Dworsky *et al.*, 1980). The estimated incidence of varicella embryopathy is <1%, with most damage due to maternal infection acquired in the first 20 weeks of gestation. The congenital varicella syndrome is most often recognized by unusual cutaneous defects and atrophy of an extremity. Infants often have microcephaly, cortical atrophy and intracranial calcifications secondary to intrauterine VZV encephalitis, with seizures and mental retardation. Damage to the autonomic nervous system is common and produces severe gastroesophogeal reflux and neurogenic bladder, hydroureter and hydronephrosis. Eye damage, manifesting as chorioretinitis, microophthalmia, and cataracts, is typical. Although intrauterine damage is not observed, infants whose mothers develop primary VZV infection just before delivery often develop varicella during the newborn period (Preblud *et al.*, 1985). The risk of transfer of virus to the infant is highest when maternal infection begins 4 days before to 2 days after delivery, suggesting that the virus crosses the placenta during the viremia associated with lesion formation. Because of the early stage of the maternal infection, viral transfer is not associated with transplacental transport of maternal VZV IgG antibodies. Neonatal varicella can be progressive, presumably due to deficiencies in the capacity of the infant to develop VZV-specific T cell responses. Dissemination causes pneumonia and hepatitis, with a risk of meningoencephalitis. These infants require antiviral therapy to prevent such complications. Infants exposed to late gestation maternal varicella can be protected to some extent by administration of passive antibodies, given as varicella immune globulin. Some infants whose mothers have varicella more than 4–5 days before delivery are born with varicella lesions or develop lesions within a few days after birth; these infants appear to be at low risk for complications. Herpes zoster in pregnant women has not been associated with varicella embryopathy.

Summary

The principal host cell targets during the life cycle of VZV include the respiratory mucosal epithelium as a portal of entry, immune system cells, especially T-cells, for delivery of the virus to skin sites of replication, and sensory ganglia, where latency is established. VZV transmission to susceptible hosts is ensured by the release of cell-free virus into mucocutaneous lesions during varicella or herpes zoster. Like HSV-1 and HSV-2, VZV is an alphaherpesvirus that has achieved an equilibrium with the human host that has ensured its persistence in the species for millions of years.

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38

VZV: molecular basis of persistence (latency and reactivation)

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Primary infection with varicella-zoster virus (VZV) causes varicella manifested by fever and a vesicular rash. During primary infection the virus disseminates in lymphocytes to the skin and other organs, and replicates in and establishes a latent infection in the nervous system (Croen *et al.*, 1988). Early studies demonstrated viral DNA in human trigeminal and dorsal root ganglia by in situ hybridization and Southern blotting (Gilden *et al.*, 1983, 1987; Hyman *et al.*, 1983). More recent studies, using PCR, have demonstrated latent VZV in multiple cranial nerve, dorsal root, and autonomic ganglia (Furuta *et al.*, 1992, 1997; Gilden *et al.*, 2001; Mahalingham *et al.*, 1990). The virus can reactivate from these sites to cause herpes zoster.

The structure of the VZV genome during latency is not certain. Clarke *et al.* (1995) performed PCR on human ganglia DNA using pairs of primers specific for the unique long internal and terminal regions of the genome. Analysis of the ratio of the signals of the PCR products indicated that the termini of the genome are adjacent during latency, suggesting that the VZV genome is probably episomal.

Site of VZV latency

A number of studies have attempted to identify the cell type in which VZV is latent in human ganglia (Table 38.1). While early studies using in situ hybridization suggested that the virus was present in neurons (Hyman *et al.*, 1983; Gilden *et al.*, 1987), other studies suggested that viral RNA was latent exclusively in satellite cells that surround the neurons (Croen *et al.*, 1988). Lungu and colleagues (1995) found VZV nucleic acid in both satellite cells and neurons.

Further studies by Dueland *et al.* (1995) and Kennedy *et al.* (1998, 1999) using in situ PCR showed that VZV is latent predominantly in neurons. LaGuardia *et al.* (1999) isolated human trigeminal ganglia, fixed the tissue, minced it, treated it with collagenase, and filtered the cells through various pore sizes of nylon mesh to separate neurons from non-noneuronal cells. PCR analysis of the neurons and non-neuronal cells showed that VZV DNA was present only in neurons. Levin and colleagues (2003) purified neurons and satellite cells and found VZV in 1.5% of neurons and in none of over 20 000 satellite cells tested. Additional studies using antibodies to VZV proteins expressed during latency detected viral proteins in neurons, but not in satellite cells (Mahalingam *et al.*, 1996; Lungu *et al.*, 1998). Wang *et al.* (2005) performed laser capture microdissection isolating single neurons or non-neuronal cells from human trigeminal ganglia. VZV DNA was present in neurons, but rarely if ever in non-neuronal cells. Theil *et al.* (2003) used double fluorescence in situ hybridization to detect VZV neurons from human trigeminal ganglia; rare satellite cells were positive for VZV. A few neurons were infected with both VZV and HSV.

In summary, while a number of studies have detected VZV RNA in both neurons and satellite cells, most recent studies indicate that neurons are the principal site of latency.

Quantification of VZV DNA load during latency

Initial studies to quantify the latent viral load used competitive PCR in which an internal mutant template is added to the unknown sample in the PCR mixture to determine the copy number of VZV genomes in human ganglia (Mahalingam *et al.*, 1993). Using this method Mahalingam and colleagues estimated that there were 9 to 53 copies of VZV DNA per ug of ganglion DNA which corresponds to 6 to 31 copies of VZV DNA per 10^5 ganglionic cells (Table 38.2). This number is considerably lower than the 1 000 to 10 000

Table 38.1. Site of VZV latency in human ganglia

Site	% neurons with VZV	%non-neuronal cells with VZV	Technique	Reference
Neuron	0.1–0.3	0	ISH	Hyman *et al.*, 1983
Neuron	"most"	0	ISH	Gilden *et al.*, 1987
Satellite	0	0.01–0.15	ISH	Croen *et al.*, 1988
Neuron = Satellite	5–30%	5–30%	ISH	Lungu *et al.*, 1995
Neuron > Satellite	2–5%	<0.1%	ISH	Kennedy *et al.*, 1998
Neuron > Satellite	2–5%	rare	IS-PCR	Kennedy *et al.*, 1999
Neuron	"many"	0	IS-PCR	Dueland *et al.*, 1995
Neuron	ND	0	Diss-PCR	LaGuardia *et al.*, 1999
Neuron	1.5%	0	Diss-PCR	Levin *et al.*, 2003
Neuron	4.1%	0.06	LCM	Wang *et al.*, 2005

ISH = in situ hybridization; IS PCR = in situ PCR; Diss-PCR = dissociated trigeminal cells and PCR; LCM = laser capture microdissection.

Table 38.2. Latent VZV DNA load

Copies per 10^5 cells		Ratio		
VZV DNA	HSV-1 DNA	VZV/HSV	Method	Reference
6–31		(0.003)	cPCR	Mahalingham *et al.*, 1993
	10^3 to 10^4			Efstathiou *et al.*, 1986
20–50		(0.005)	cPCR	LaGuardia *et al.*, 1999
258	2902	0.1	rPCR	Pevenstein *et al.*, 1999
9046	3042	3	rPCR	Cohrs *et al.*, 2000
38–179			cPCR	Levin *et al.*, 2003
283	711	0.4	LCM	Wang *et al.*, 2005

cPCR = quantitative DNA PCR; rPCR = real time PCR; LCM = laser capture microdissection.

copies of HSV DNA per 10^5 ganglionic cells (Efstathiou *et al.*, 1986).

LaGuardia and colleagues (1999) performed PCR on sets of 100 neurons obtained from human trigeminal ganglia; of PCR-positive sets of neurons, there were two to five copies of VZV DNA per 100 neurons. Assuming that there are 100 cells per neuron in human ganglia (Mahalingam *et al.*, 1993), these results correspond to 20–50 copies of VZV DNA per 10^5 ganglionic cells. Levin *et al.* (2003) performed PCR on purified neurons and detected an average of 4.7 copies of VZV per latently infected neuron. Since they found that 0.8%–3.8% of neurons were latently infected, this corresponds to 3.8–17.9 copies per 100 neurons or 38–179 copies per 10^5 ganglionic cells.

Pevenstein *et al.* (1999) used real time PCR to determine the number of copies of VZV and HSV-1 DNA in human trigeminal ganglia. They estimated that the copy number of VZV DNA was 177 to 299 per 10^5 ganglion cells, while the copy number of HSV-1 DNA was 2902 per 10^5 ganglia cells. This suggests that there is about tenfold more HSV-1 DNA than VZV DNA in latently infected ganglia.

Cohrs *et al.* (2000) also used real time PCR to estimate copy numbers in human trigeminal ganglia. The mean VZV DNA copy number was 580 copies per 100 ng of ganglia DNA (corresponding to 9,046 copies per 10^5 cells) and the mean HSV-1 DNA copy number was 195 per 100 ng of DNA (corresponding to 3,042 copies per 10^5 cells). This suggests that there is about a 3-fold lower amount of HSV-1 than VZV DNA in latently infected human ganglia.

Wang *et al.* (2005), using laser capture microdissection to measure VZV DNA in trigeminal ganglia from 10 subjects, found a median of 6.9 copies of VZV per positive neuron. Since 4.1% of neurons were VZV positive, and there are about 100 cells per neuron in human ganglia, this corresponds to a median of 283 VZV DNA copies per 10^5 ganglionic cells. The same authors measured HSV DNA in ganglia from 6 subjects and found a median of 11.3 copies of HSV per positive neuron and 6.3% of neurons were positive of HSV. This corresponds to a median of 711 HSV copies per 10^5 cells.

The difference between the three studies (Cohrs *et al.*, 2000; Pevenstein *et al.*, 1999; Wang *et al.*, 2005) may be due to the difference in age of the subjects; those in the study by Cohrs *et al.* were older and may have had more episodes of asymptomatic reactivation with increased seeding of ganglia with VZV DNA. Alternatively, the differences may be due to the relatively small number of subjects tested (<20) in each study and the wide range in the amount of VZV DNA detected in ganglia from different subjects.

Animal models for VZV latency

Inoculation of several rodent species with VZV results in a latent infection of the nervous system. VZV was detected in trigeminal and thoracic ganglia from hairless guinea pigs by PCR after inoculation with a guinea pig adapted strain of virus (Lowry *et al.*, 1993). VZV DNA was present in mouse trigeminal ganglia 1 month after corneal inoculation with the virus (Wroblewska *et al.*, 1993). Inoculation of adult rats with VZV in the footpad or by paraspinal intramuscular injection results in latent virus infection in the dorsal root ganglia (Debrus *et al.*, 1995; Sadzot-Delvaux *et al.*, 1990; Annunziato *et al.*, 1998). Using this system, Sadzot-Delvaux

and colleagues (1995) found that viral DNA was present as early as 2 days after infection and up to 9 months later. In most animals, only the ganglia innervating the inoculation site were latently infected, indicating that ganglia were infected by retrograde transport of virus up the axon, rather than by viremia. Up to 80% of neurons were infected; some non-neuronal cells were also infected. The pattern of latent viral transcription and protein expression was similar in rats to that seen in humans. Kennedy and colleagues (2001) were able to detect latent transcripts in rat ganglia up to 18 months after inoculation. Sato *et al.* (2002b) inoculated cotton rats with VZV and obtained latent infection, based on DNA and RNA transcripts, in dorsal root ganglia of 50% to 70% of animals. Inoculation of animals with heat-inactivated virus-infected cells failed to result in latency. VZV was not detected in the lungs and usually not in the brain of animals (Sato *et al.*, 2003b). Brunell and colleagues (1999) inoculated neonatal rats with an early passage isolate of VZV intraperitoneally, and detected a latent viral transcript in trigeminal ganglia.

Baiker *et al.* (2004) transplanted fetal human brain stem cells into NOD-SCID mice and then inoculated the animals with VZV-infected melanoma cells. VZV infected both neural and glial cells. While latency-associated proteins (ORF62, ORF63, ORF47 proteins) were detected and a late protein not associated with latency (gE) was rarely detected in the animals, the localization of the latency-associated proteins was either in the nucleus or in the nucleus and the cytoplasm, unlike that seen in latently infected human neurons (see below). Zerboni *et al.* (2005) implanted human fetal dorsal root ganglia under the capsule of the kidney of SCID mice and then either injected the implants with VZV-infected fibroblasts or injected the animals intravenously with VZV-infected tonsil T cells. Direct injection of the ganglia resulted in a lytic infection followed by latency with expression of the ORF63 latency gene, but not the ORF62 latency gene or gB 8 weeks after infection. Intravenous injection of T cells resulted in a lytic infection in the ganglia 2 weeks after injection.

Simian varicella virus (SVV) is the simian counterpart of VZV, and the two viruses share nearly all the same open reading frames (Gray *et al.*, 2001; Gray, 2004). Intratracheal inoculation of SVV into African green monkeys results in infection of the trigeminal and dorsal root ganglia (Mahalingam *et al.*, 1991); however, other sites including peripheral blood mononuclear cells and lungs show persistent infection (White *et al.*, 2002). Naturally acquired SVV infection, transmitted from one African green monkey to another, results in a mild rash followed by latent infection of multiple ganglia, but not infection of other tissues (Mahalingam *et al.*, 2002). SVV DNA is present only in neurons of latently infected monkeys (Kennedy *et al.*, 2004) At least one VZV gene shown to be expressed during latency, ORF21, is expressed in ganglia from monkeys infected with SVV (Clarke *et al.*, 1996). The SVV model is the only animal model for VZV that recapitulates the features of varicella, latency, and reactivation.

Table 38.3. VZV transcripts consistently expressed during latency[a]

Transcript	Species	Method	Reference
4 (IE)[b]	human	ISH	Kennedy *et al.*, 1999; Kennedy *et al.*, 2000
	rat	Northern & PCR	Sadzot-Delvaux *et al.*, 1995
21 (E)	human	cDNA library	Cohrs *et al.*, 1996
	human	real time PCR	Cohrs *et al.*, 2000
	human	ISH	Kennedy *et al.*, 1999; Kennedy *et al.*, 2000
	neonatal rat	RT-PCR	Brunell *et al.*, 1996
	rat	ISH	Kennedy *et al.*, 2001
29 (E)	human	Northern	Meier *et al.*, 1993
	human	cDNA library	Cohrs *et al.*, 1996
	human	real time PCR	Cohrs *et al.*, 2000
	human	ISH	Kennedy *et al.*, 1999; Kennedy *et al.*, 2000
	rat	Northern & PCR	Sadzot-Delvaux *et al.*, 1995
	rat	ISH	Kennedy *et al.*, 2001
62 (IE)	human	Northern	Meier *et al.*, 1993
	human	cDNA library	Cohrs *et al.*, 1996
	human	ISH	Kennedy *et al.*, 2000
	rat	Northern & PCR	Sadzot-Delvaux *et al.*, 1995
	rat	ISH	Kennedy *et al.*, 2001
	human	FISH	Thiel *et al.*, 2003
63 (IE)	human	cDNA library	Cohrs *et al.*, 1996
	human	real time PCR	Cohrs *et al.*, 2000
	human	ISH	Kennedy *et al.*, 1999; Kennedy *et al.*, 2000
	rat	Northern & PCR	Sadzot-Delvaux *et al.*, 1995
	rat	ISH	Kennedy *et al.*, 2001
	cotton rat	RT-PCR	Sato *et al.*, 2002b
66 (E)	human	ISH, RT-PCR	Cohrs *et al.*, 2003

[a]Generally not expressed during latency: 10 (L), 14 (L), 28 (E), 36 (E), 40 (L), 61, 67 (L), 68 (L).
[b]Predicted temporal class of transcription: immediate-early (IE), early (E), late (L).

VZV transcripts expressed during latency

A number of laboratories have detected transcripts corresponding to various VZV genes in human ganglia and from experimentally infected rodents (Table 38.3). Meier *et al.* (1993) detected transcripts for VZV ORF 29 and ORF62, but

Table 38.4. Latent VZV and HSV-1 RNA loads in human trigeminal ganglia[a]

VZV RNA Transcript	Median Copy Number[b] (range)	% Positive Ganglia	Median VZV DNA Copy Number[c] (range)	% Positive Ganglia
21	6.2 (0.6–85.5)	23%	223 (24.9–4027)	100%
29	427 (56–1,154)	13%		
63	121 (17–2,786)	86%		
HSV RNA Transcript	Median Copy Number (range)	% Positive ganglia	Median HSV-1 DNA copy number (range)	% Positive ganglia
LAT	1188 (44–23,070)	68%	162 (22.5–289)	68%

[a]Data from Cohrs *et al.*, 2000.
[b]Expressed as number of virus gene transcripts/number of GADPH transcripts $\times 10^4$.
[c]Expressed per 100 ng of DNA.

Table 38.5. VZV Proteins expressed during latency[a]

Protein	Species	Method	Reference
4 (IE)[b]	human	IH	Lungu *et al.*, 1998
62 (IE)	human	IH	Lungu *et al.*, 1998; Kennedy *et al.*, 2004
63 (IE)	rat	IH	Debrus *et al.*, 1995; Kennedy *et al.*, 2001
	human	IH	Mahalingham *et al.*, 1996; Lungu *et al.*, 1998; Kennedy *et al.*, 2000; Kennedy *et al.*, 2004
21 (E)	human	IH	Lungu *et al.*, 1998; Kennedy *et al.*, 2004
29 (E)	human	IH	Lungu *et al.*, 1998; Kennedy *et al.*, 2004
66 (E)	human	IH	Cohrs *et al.*, 2003

[a]VZV proteins ORF 10 (L), 14 (L), 67 (L) not detected.
[b]Predicted temporal class of transcription: immediate-early (IE), early (E), late (L).

not ORF28 by Northern blot analysis of RNA extracted from pooled human trigeminal ganglia.

Cohrs *et al.* (1996) detected VZV ORFs 21, 29, 62, and 63 from cDNA obtained by isolating RNA from pooled human trigeminal ganglia. They were unable to detect transcripts for ORFs 4, 10, 40, 51, and 61 in the library. ORF66 was also detected in latently infected human ganglia (Cohrs *et al.*, 2003).

Kennedy *et al.* (1999, 2000) used in situ hybridization and detected VZV transcripts for ORFs 21, 29, 62, and 63 during latency. ORF21 transcripts were detected in 64% of ganglia from HIV-negative subjects and in 60% from those with HIV; ORF29 transcripts were found in 38% of ganglia from HIV-negative persons and in 100% from those with HIV; ORF62 transcripts were noted in 40% of ganglia from HIV-negative subjects and in 67% from those with HIV; ORF63 transcripts were detected in 47% of transcripts from HIV-negative persons and in 80% from those with HIV. Transcripts for VZV ORFs 4 and 18 were infrequently expressed, while those for VZV ORFs 28, 40, and 61 were rarely expressed.

Cohrs *et al.* (2000) used real time PCR to detect transcripts for VZV ORF 63 from 86% of human trigeminal ganglia assayed. Transcripts for ORFs 21 and 29 were detected less frequently in 23% and 13% of ganglia, respectively. While the copy numbers for ORF63 transcripts varied over a wide range, they were consistently lower than the copy numbers for HSV-1 LAT transcripts from the same ganglia (Table 38.4).

Several studies have looked at rodents (adult rats, neonatal rats, and adult cotton rats) latently infected with VZV (Table 38.3). Transcripts for ORFs 4, 21, 29, 62 and 63 have been detected in these animals (Kennedy *et al.*, 2001; Sadzot-Delvaux *et al.*, 1990; Brunell *et al.*, 1999; Sato *et al.*, 2002b). Transcripts for ORFs 4, 28, 36, 40, and 68 were not detected. VZV gene expression during latency may be regulated by epigenetic mechanisms. Gary *et al.* (2006) showed that the VZV ORF62 and 63 promoters are associated with a histone protein (acetylated H3K9) and thereby maintained in a euchromatic state during latency and thus can be transcribed. In contrast, the ORF14 and 36 promoters whose genes are not expressed during latency are not associated with the histone protein and instead are maintained in a heterochromatic state.

In summary, a number of studies from several different laboratories have detected 6 different viral transcripts during latency in humans and in rodents. These are predicted to encode immediate-early and early proteins. In contrast, other putative early (ORFs 28, 36, 51) and late (ORFs 10, 40, 68) transcripts have not been found in latently infected human or rodent ganglia.

VZV proteins expressed during latency

Six VZV proteins have also been detected during latency (Table 38.5). Three laboratories (Mahalingham *et al.*, 1996; Kennedy *et al.*, 2000, 2004; Lungu *et al.*, 1998) have detected ORF63 protein in human ganglia using antibody to the

protein. In all four studies, ORF63 protein was found either predominantly or exclusively in the cytoplasm of neurons. This is in contrast to its usual localization in both the nucleus and cytoplasm early during lytic infection, and in the nucleus late in lytic infection in vitro (Debrus *et al.*, 1995).

Debrus *et al.* (1995) and Kennedy *et al.* (2001) inoculated rats in the footpad with VZV and detected ORF63 protein in lumbar ganglia. ORF63 protein was present in both the cytoplasm and nucleus of neurons using either a polyclonal (Debrus *et al.*, 1995) or monoclonal (Kennedy *et al.*, 2001) antibody to the protein.

In addition to ORF63 protein, ORFs 4, 21, 29, and 62 proteins were detected exclusively in the cytoplasm of neurons of latently infected human ganglia using polyclonal antibodies in one study (Lungu *et al.*, 1998) and ORFs21, 29, and 62 proteins were detected predominantly in the cytoplasm of neurons in another study (Kennedy *et al.*, 2004). During lytic infection in vitro, ORF21, 29, and 62 proteins are present predominantly in the nucleus, while ORF4 protein is present mostly in the cytoplasm. Lungu *et al.* (1998) postulated that these proteins are sequestered from the nucleus during latency and cannot function to transactivate genes or replicate viral DNA. VZV ORF 10, 14, and 67 proteins were not detected by the authors in human ganglia. Cohrs *et al.* (2003) detected ORF66 protein exclusively in the cytoplasm of neurons.

Function of VZV latency-associated proteins

Three of the VZV genes expressed during latency (ORFs 4, 62, 63) have been identified as immediate-early proteins. ORF62 protein is the major viral transactivator and up-regulates expression of all VZV genes that have been tested in transient expression assays (reviewed in Cohen and Straus, 2001). ORF4 protein transactivates expression of certain VZV genes and enhances the ability of ORF62 to transactivate VZV gene expression. VZV ORF63 down-regulates expression of ORF62 transcripts and protein (Hoover *et al.*, 2006). Cells infected with VZV ORF63 deletion mutants that are impaired for latency show an increase in ORF62 transcription relative to those infected with parental virus, while cells infected with ORF63 mutants not impaired for latency show levels of ORF62 transcription similar to parental virus. Thus, expression of ORF63 during latency may allow the cell to down-regulate ORF62 transcription and limit lytic gene expression. ORF63 protein may have a role in inhibiting apoptosis. Expression of ORF63 protein in rat neurons inhibited apoptosis induced by withdrawal of nerve growth factor (Hood *et al.*, 2006); however, ganglia from cotton rats acutely infected with an ORF63 deletion mutant did not show an increase in apoptosis (Cohen *et al.*, 2004). ORF4, ORF62, and ORF63 proteins are all present in the viral tegument.

VZV ORF29 is predicted to encode an early protein, while ORFs 21 and 66 are predicted to encode late proteins, based on their homology with HSV genes. VZV ORF21 protein is present in the viral nucleocapsid and ORF29 protein is a single-stranded DNA binding protein that regulates gene expression from the gI promoter. ORF66 encodes a serine-threonine protein kinase that phosphorylates the ORF62 protein. The actual function of these six VZV genes during latent infection is unknown.

VZV genes required for establishment of latent infection

The cotton rat model has been used to test the ability of several VZV mutants to establish a latent infection. VZV ORFs 1, 2, 13, 32, and 57 encode proteins that do not have HSV homologues. Inoculation of cotton rats with VZV mutants unable to express each of these proteins showed that each is dispensable for establishment of latency (Sato *et al.*, 2002b, 2003b). VZV ORF10, ORF14, ORF17, ORF61, and ORF67 encode the homologues of the HSV VP16 transactivator, glycoprotein C, viral host shut off protein, ICP0 immediate-early protein, and glycoprotein I, respectively. All five of these VZV proteins are dispensable for latency in the cotton rat (Kennedy *et al.*, 2004; Sato *et al.*, 2002a, 2003a, b). ORF21 and ORF66 proteins, which are expressed during VZV latency, are also dispensable for establishment of latent infection (Sato *et al.*, 2003b; Xia *et al.*, 2002). VZV ORF47 protein, which is required for infection of human T cells and skin is dispensable for latency. In contrast, deletion of ORF4 or ORF63 results in a virus that is impaired for establishment of latency (Cohen *et al.*, 2004, 2005b).

In vitro models for VZV latency

Several investigators have infected human neurons with VZV in vitro in attempts to produce models for latency. Infection of human fetal dorsal root ganglia neurons with cell associated or cell-free VZV resulted in a productive infection, but neurons were less susceptible to infection or had a slower rate of infection than non-neuronal cells (Wigdahl *et al.*, 1986; Assouline *et al.*, 1990). Somekh and colleagues (1992) prepared human fetal neuron, satellite cell, or mixed neuron and satellite cell cultures from dorsal root ganglia and infected the cells with cell-free VZV in the presence of BVaraU. After 1 week BVaraU was removed, and 1 to 3 weeks later the cells were plated onto human

fibroblasts. VZV "reactivated" from 56% of mixed cultures (neurons and satellite cells), but not from pure neuron or satellite cell cultures.

Merville-Louis *et al.* (1989) infected adult rat dorsal root ganglia neurons with cell associated or cell-free VZV and observed all three kinetic classes of VZV transcripts in the cells; CPE was not observed and virus was not produced. This suggests that the cells underwent an abortive, but not latent infection. In contrast, Kress *et al.* (2001) infected adult rat dorsal root ganglia with cell-associated virus and noted productive infection with death of the cells. Hood *et al.* (2003) infected human dorsal root ganglion neurons with cell-associated VZV and observed expression of all three kinetic classes of VZV proteins, but apoptosis did not occur, in contrast to the marked apoptosis observed in virus-infected fibroblasts.

Chen *et al.* (2003) infected guinea pig enteric neurons with cell-free or cell-associated virus. Infection with cell-associated virus resulted in a productive infection of the neurons, while infection with cell-free virus resulted in a latent pattern of infection. The latently infected neurons expressed VZV ORF4, 21, 29, 62, and 63 proteins in the cytoplasm of the cells; however, glycoproteins were not expressed. The authors postulated that cell-free VZV, unlike cell-associated virus, lacks some of the proteins that are present in virus-infected cells at the onset of infection and therefore is not able to initiate virus replication in neurons. Stallings *et al.* (2006) showed that while ORF29 and ORF62 proteins are excluded from the nucleus of cultured guinea pig enteric neurons infected with VZV, expression of VZV ORF61 protein in these cells resulted in translocation of ORF29 and ORF62 proteins to the nucleus. These studies suggest that VZV ORF61 protein may contribute to reactivation of virus from latency.

While these in vitro models recapitulate some of the features of latent VZV infection, it is not clear how well they emulate latency in vivo. The limitations of cell culture based models of HSV latency to simulate latency in vivo, and the lack of understanding of what actually constitutes latency in vivo, suggests that in vitro models for VZV may also have limitations.

Reactivation of VZV

VZV reactivation, which presents as zoster, occurs more frequently in immuncompromised persons and in the elderly. A decline in the frequency of VZV-specific T-cells is thought to allow the virus to clinically reactivate. VZV-specific cytotoxic T-lymphocytes (CTLs) have been detected that recognize the ORF4, 29, 62, and 63 latency proteins (Sadzot-Delvaux *et al.*, 1997; Arvin *et al.*, 2002). These CTLs may help to prevent reactivation of VZV from latency. The demonstration that vaccination of adults with varicella-zoster virus vaccine can reduce the incidence of zoster and postherpetic neuralgia, suggests that augmentation of the cellular immune response to VZV can reduce reactivation (Oxman *et al.*, 2005).

The molecular basis of reactivation is not known. While HSV can be cocultivated from human ganglia, infectious VZV has never been recovered from human ganglia. Vafai *et al.* (1988) cultured human ganglia in tissue culture media for 11 to17 days and detected 7 major VZV-specific proteins of 35 to 200 kDa. While these proteins were not identified, lysates from the cultivated ganglia did not react with monoclonal antibodies to VZV proteins expressed during the late phase of the replicative cycle, indicating that viral replication did not occur in vitro. Kennedy *et al.* (2000) studied human trigeminal and dorsal root ganglia that were cocultivated with monkey kidney cells for 3 to 11 days. Transcripts associated (ORFs 29 and 63) and not associated (ORFs 18, 28, and 40) with latency were detected in most of the ganglia. This pattern was very different from that observed in ganglia that were not cocultivated, suggesting that reactivation had occurred. No evidence of infectious VZV was detected after cocultivation. In one report, VZV was said to have reactivated, as detected by cytopathic effects and in situ hybridization, from VZV-infected rats after cocultivation of dissociated ganglia with human fibroblasts and repeated treatment of the cells with trypsin (Sadzot-Delvaux *et al.*, 1990). This finding has not yet been confirmed by others.

Analysis of VZV DNA in dorsal root ganglia from a person with zoster showed that viral DNA was present both in neurons and satellite cells of ganglia innervating the sites of reactivation (Lungu *et al.*, 1995). VZV DNA was present in both the nucleus and the cytoplasm of reactivating ganglia, and a late viral protein, gI, was present in the cytoplasm of these ganglia. A subsequent study showed that while latency associated proteins were present in only the cytoplasm during latency, these proteins translocated to both the cytoplasm and nucleus of neurons in ganglia undergoing reactivation (Lungu *et al.*, 1998). Latency associated proteins were not detected in the satellite cells of the ganglia.

Comparison of VZV latency with that of other alphaherpesviruses

VZV latency has a number of different features than that seen in HSV (Table 38.6). During latency of other alphaherpesvirus, such as HSV, bovine herpesvirus (BHV), or pseudorabies virus (PRV), only the latency associated

transcripts (LAT) are expressed. The BHV LAT encodes a protein, while no HSV LAT protein has been detected in latently infected humans or animals. PRV, like VZV, is a member of the varicellovirus family, however, only the PRV LAT transcript has been detected during latency. At present it is unclear why latent gene expression in VZV is different from that of the other alphaherpesviruses.

Is the large number of transcripts in VZV latency due to reactivation?

A number of observations suggest that the large number of VZV transcripts that are detected during latency in humans is not an artifact due to reactivation. First, comparison of transcripts detected from human ganglia shortly after death, with those obtained after ganglia are explanted show different patterns of gene expression (Kennedy *et al.*, 2000). Second, similar studies in which human ganglia are obtained after death detect only a single latency-associated transcript for HSV-1 (Croen *et al.*, 1988). Since HSV can undergo reactivation with productive infection in cell culture, apparently the brief period of time between death and processing the ganglia to detect latent transcripts is insufficient to induce reactivation and may also hold true for VZV. Third, the time between death and obtaining tissue at autopsy did not correlate with the number of VZV genome copies (Cohrs *et al.*, 2000). Fourth, only certain immediate-early and early VZV genes are expressed during latency. Finally, animal models of VZV, in which ganglia are processed immediately after death, show a similar pattern of VZV transcription and protein expression during latency as occurs in human ganglia (Sadzot-Delvaux *et al.*, 1995).

Models for VZV latency

The relatively large number of transcripts that are expressed during latency along with the presence of viral proteins, in latently infected neurons indicates that the mechanism of latency for VZV differs from that of herpes simplex virus. Two models may be proposed to explain the ability of the virus to remain latent, despite expression of several gene products.

VZV proteins localize to the cytoplasm, instead of the nucleus of neurons and thus are unable to carry out their activities

VZV ORF21, 29, 62, and 63 proteins, are usually present in the nucleus of cells during lytic replication. Four studies (Mahalingam *et al.*, 1996; Grinfeld and Kennedy, 2004; Kennedy *et al.*, 2000; Lungu *et al.*, 1998) have shown that ORF63 is located in the cytoplasm of latently infected neurons. Furthermore, Lungu and colleagues (1998) detected ORF21, 29, and 62 proteins exclusively in the cytoplasm of latently infected neurons. Segregated to the cytoplasm, ORF62 protein would not be able to transactivate VZV promoters, and ORF29 protein would not be able to bind to single-stranded DNA to perform its regulatory activities during DNA replication. While the functions of ORF21 and ORF63 are not known, their sequestration in the cytoplasm during latency, away from the nucleus, suggests that they might be unable to carry out their activities during latency.

How might these latency-associated proteins be sequestered in the cytoplasm during latency? Bontems *et al.* (2002) showed that the phosphorylation status of IE63 protein determines its localization in either the cytoplasm or nucleus. Transient expression of wild-type ORF63 protein resulted in a predominantly nuclear localization in Vero cells, while mutation of several serine or threonine residues that are sites of phosphorylation resulted in a predominantly cytoplasmic localization. While the phosphorylation pattern of ORF63 protein may appear to regulate its localization, the same authors showed that ORF63 protein localized to the nucleus of a neural cell line regardless of its phosphorylation status. In addition, localization of different phosphorylated forms of ORF63 is apparently dependent on whether the protein is expressed alone (Bontems *et al.*, 2002) or in the context of the rest of the genome (Cohen *et al.*, 2005a)

Table 38.6. Differences between VZV and HSV-1 infection, latency, and reactivation

Property	VZV	HSV-1
Primary infection	Disseminated	Localized
Entry into ganglia	Viremia, neural	Neural
Frequency of reactivation	Usually once	Multiple
Likelihood of reactivation	Increases with age	Decreases with age
Asymptomatic reactivation with virus shedding	No	Frequent
Distribution	Dermatome (ganglia)	Focal (sensory nerve)
Reactivation stimuli	None	UV, fever
Reactivation symptoms	Severe pain	Mild or none
Time to recurrence after immunosuppression	2–6 months	1–4 weeks
Latency associated RNAs and proteins	ORFs4, 21, 29, 62, 63, 66 RNA and protein	LAT RNA only

The phosphorylation status of ORF62 protein also regulates its localization (Kinchington *et al.*, 2000). ORF66 protein phosphorylates ORF62 protein and keeps it sequestered to the cytoplasm. Since ORF66 is expressed during latency, this might be a mechanism to keep ORF62 protein from activating transcription during latency. However, a VZV mutant that is unable to express ORF66 still maintains latent infection in an animal model (Sato *et al.*, 2003b). Thus, there are likely to be factors in addition to phosphorylation that are important for inhibiting the lytic functions of ORF63 and ORF62 protein during latency.

This model still leaves a number of questions unanswered. It is unclear from this model how ORF 21 and ORF29 are expressed if ORF62 is sequestered in the cytoplasm. Furthermore it is unclear why gene expression is limited to selected immediate-early and early genes and not other early or late genes.

VZV proteins have different activities in neurons than in permissive cells due to differences in cellular proteins

Differences in cellular proteins, such as transcription factors, may be responsible for the different activity of VZV in latently infected neurons compared to permissive cells. ORF29 protein augments the ability of ORF62 protein to transactivate the gI promoter in fibroblasts and T-cells; however, ORF29 inhibits the ability of ORF62 protein to activate the gI promoter in neuronal (PC-12) cells (Boucaud *et al.*, 1998). Similarly, ORF63 protein activates the EF-1α promoter in melanoma cells, but not in neuroblastoma cells (Zuranski *et al.*, 2005). This suggests that either neuronal cells lack an activator or that these cells express a repressor that is lacking in fibroblasts and T cells.

Neuronal cells may lack transcription factors, or may have transcription factors that are in an inactive form. VZV ORF10 forms a complex with TAATGARAT-like elements, Oct1 and host cell factor (HCF) to transactivate the ORF62 promoter (Moriuchi *et al.*, 1995). HCF is sequestered in the cytoplasm in sensory neurons, but translocates to nucleus during stimuli that induce reactivation of HSV (Kristie *et al.*, 1999). If HCF is important for reactivation of VZV, its normal sequestration in the cytoplasm may help VZV to maintain a latent infection.

Alternatively, transcription factors may act as repressors in neurons, but not in permissive cells. Transient expression Oct 2 in hamster kidney cells (in which the protein is normally not expressed) increases the basal activity of the ORF62 promoter (Patel *et al.*, 1998). In contrast, expression of Oct2 in a neuronal cell line represses the activity of the ORF62 promoter. Since Oct2 is normally expressed only in neuronal and B-cells, Oct2 may repress the ORF62 promoter in these cells and help to maintain latency.

Oct 2 can also inhibit transactivation of the ORF62 promoter by the ORF10 protein. Transient expression of Oct2 with the ORF10 transactivator and an ORF62 promoter driving CAT as a reporter construct inhibited the ability of ORF10 protein to transactivate the ORF62 promoter. Thus, expression of Oct2 in neurons may inhibit the transactivating activity of ORF10, which might otherwise stimulate reactivation of VZV in neurons.

Future directions

While the last several years have resulted in much new information about VZV latency, many issues still remain. Further studies are needed from other laboratories to confirm the pattern of VZV protein expression during latency, preferably using monoclonal antibodies.

Additional studies using animal models will be critical in studies of VZV latency. Since animals can be sacrificed and ganglia removed quickly, reactivation is less of an issue. The simian varicella virus is a particularly attractive model since one can study acute and latent disease in the natural animal model. While the simian virus is the closest homologue to VZV, there are some differences in the genomes of the two viruses, and it is uncertain if these differences would result in different mechanisms of latency.

A number of questions regarding latency remain unanswered. At present it is unclear why VZV, unlike the other alphaherpesvirus, expresses so many viral genes during latency. Why are many of the immediate-early genes, which normally initiate VZV infection, expressed during latency? What are the molecular mechanisms involved in maintaining latency and in inducing reactivation?

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VZV immunobiology and host response

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Immunobiology

Introduction

Varicella zoster virus (VZV) like the other herpesvirus family members is a highly successful and ubiquitous human pathogen. In order for VZV to persist in the human population, the virus has evolved strategies to avoid immune detection and potentially promote viral pathogenesis. We have demonstrated that VZV encodes two separate immune evasion strategies by specifically down-regulating cell-surface MHC class I (Abendroth *et al.*, 2001a) and inhibiting the up-regulation of interferon-γ-induced MHC class II expression (Abendroth *et al.*, 2000) during productive infection of primary human foreskin fibroblasts (HFFs). Given that VZV appears to evade host recognition by T-cells during the prolonged, 10–21 day incubation period, viral genes encoding immunomodulatory proteins are likely to delay the initial clonal amplification of VZV specific $CD4^+$ and $CD8^+$ T-lymphocytes and at least transiently enhance the ability of VZV to replicate at cutaneous sites. Recently we have studied the interaction of VZV with human dendritic cells (DCs) and T-lymphocytes. VZV has the ability to infect immature DCs and transfer virus to T-lymphocytes (Abendroth *et al.*, 2001b). VZV also readily infects tonsil T-cells (Ku *et al.*, 2002). The analysis of VZV interactions with T-cells during viral pathogenesis is described in Chapter 37. These capacities of VZV to infect DC and T-cells provide new models of viral dissemination during primary and recurrent VZV infections. Further studies assessing mature DCs have revealed a third immune evasion mechanism for VZV whereby the virus is able to productively infect a specialized immune cell (representing the most potent antigen presenting cell type), and in doing so impairs its ability to function properly.

More recently we have revealed that VZV has evolved a mechanism to limit host cell anti-viral defenses by impairing NF_{KB} activation in cultured fibroblasts and in differentiated epidermal cells in Skin xenografts in SCID by mice infected in vivo (Jones and Arvind, 2006).

VZV encoded downmodulation of cell-surface MHC class I expression

Our initial studies on VZV encoded immunomodulation began with the observation that VZV could downregulate cell-surface MHC class I expression in HFFs, which are the optimal cell-type for VZV infection and replication in vitro (Arvin, 2001). Cell-surface MHC class I and VZV antigen expression was examined by flow cytometry on HFFs infected with a low passage clinical VZV isolate (strain S) (Abendroth *et al.*, 2001a). In cells where no viral antigen was observed, approximately 70%–80% of cells had detectable MHC class I expression, whereas only about 20%–30% of the cells that had detectable VZV antigen synthesis also expressed cell-surface MHC class I. VZV selectively downregulated cell-surface MHC class I expression on HFFs as flow cytometry of these cells for transferrin receptor (CD71) expression, revealed that VZV infection did not alter cell-surface CD71 expression (Fig. 39.1). Cohen and co-workers have also found that infection of human fibroblasts with VZV results in the specific downregulation of cell-surface MHC class I expression (Cohen, 1998). We have further demonstrated that VZV isolates, whether fresh clinical isolates or tissue-culture passaged virus, specifically downregulate MHC class I expression in primary and transformed human cells.

Given this evidence for MHC class I down-regulation following VZV infection of tissue culture cells, we sought to determine the potential biological significance of this effect in facilitating VZV pathogenesis. Based on our previous

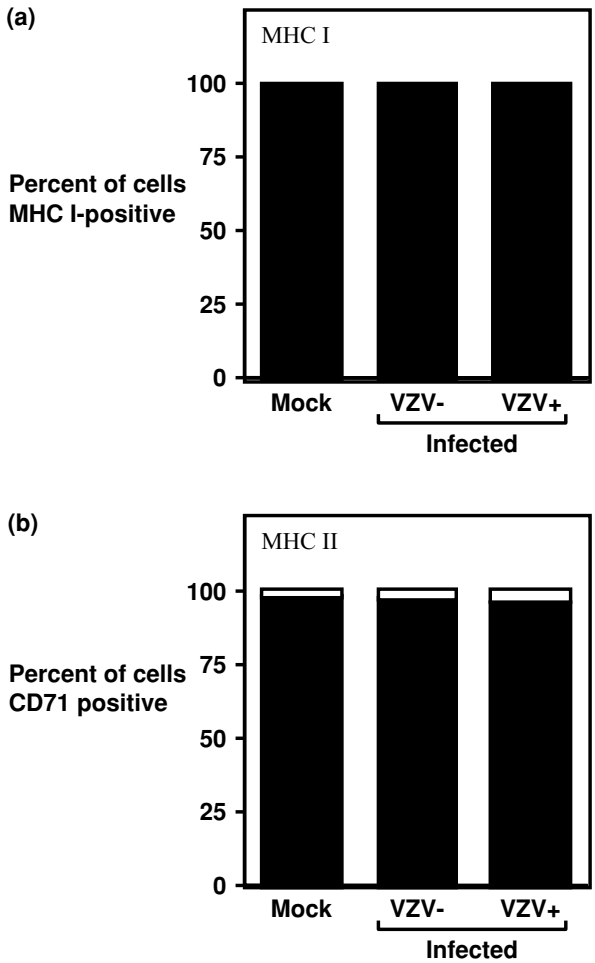

Fig. 39.1. FACS analysis of MHC class I, transferrin receptor (CD71) and VZV proteins on VZV infected cells. HFF were either infected with VZV for 24 hr or mock infected, cell preparations were stained with antibodies and fluorescent conjugates to MHC I and VZV proteins or to transferrin receptor (CD71) and VZV proteins. The percentage of VZV^{+} and VZV^{-} cell populations expressing cell surface MHC I and transferrin receptor is shown using analysis by flow cytometry.

studies showing that VZV causes cell-associated viremia during the incubation period of primary infection without inducing a detectable immune response, we evaluated whether VZV infection altered MHC class I expression on human T-cells in vivo using the SCID-hu mouse model. We have previously demonstrated that VZV infects human CD4+ and CD8+ T-cells as well as immature CD4+CD8+ in SCID-hu thymus/liver implants *in vivo* (Moffat *et al.*, 1995). To determine whether VZV infection alters MHC class I expression on human T-cells, human fetal thymus/liver implants placed under the kidney capsule of SCID-hu mice were inoculated with VZV according to our standard protocol (Moffat *et al.*, 1995). Cells from the infected and uninfected implants were immunostained with antibodies to the CD3 T-cell marker, MHC class I and VZV proteins and analysed by flow cytometry. In all mice inoculated with VZV, CD3 positive T-cells expressing viral antigens were readily detectable (range of 11%–20%). The percentage of T-cells that expressed MHC class I within the population of cells that was positive for both CD3 and VZV proteins was significantly decreased when compared to the population that was positive for CD3 but had no detectable VZV proteins. Thus, VZV replication has the capacity to cause MHC class I down-regulation on human CD3 T cells a cell-type critical for the viremic phase of VZV pathogenesis. This observation is consistent with the concept that this immunomodulatory mechanism may provide the virus with a transient advantage, allowing viral dissemination to skin sites of replication that are required to achieve transmission.

Recently, we identified the first VZV immunomodulatory gene product and described the mechanism by which VZV alters cell-surface MHC class I expression (Abendroth *et al.*, 2001a). To identify the compartment of MHC class I retention within the cell, we assessed the localization of MHC class I complexes with organelle specific markers in uninfected and VZV infected cells by confocal microscopy. Uninfected HFFs showed a uniform distribution of MHC class I throughout the cell cytoplasm and surface. In contrast, VZV infected HFs showed a strong perinuclear staining pattern for MHC class I molecules that colocalized with ceramide which labelled the membranes of the Golgi complex. The intracellular localization of MHC class I molecules to the Golgi in VZV infected cells suggests that a viral protein may interact specifically with a component of the MHC class I complex, thereby preventing efficient MHC class I transport to the cell-surface. In this respect, other herpesviruses, including HSV and human and murine CMV, have been shown to encode immunomodulatory proteins that directly associate with components of the MHC class I biosynthesis pathway (York *et al.*, 1994; Fruh *et al.*, 1995; Hill *et al.*, 1995; Ahn *et al.*, 1996; Jones *et al.*, 1996; Tomazin *et al.*, 1996; Wiertz *et al.*, 1996; Galocha *et al.*, 1997; Jones and Sun, 1997; Kleijnen *et al.*, 1997; Machold *et al.*, 1997; Reusch *et al.*, 1999). Immunoprecipitation of radiolabelled MHC class I molecules in VZV and mock infected cells did not reveal binding of a viral protein but a modified, ~40 KDa MHC class I was detected consistently in VZV infected cells, which may reflect accelerated degradation of MHC class I molecules retained in the Golgi.

Despite the sequence similarities between the VZV and HSV genomes, VZV does not encode an ICP47 homologue.

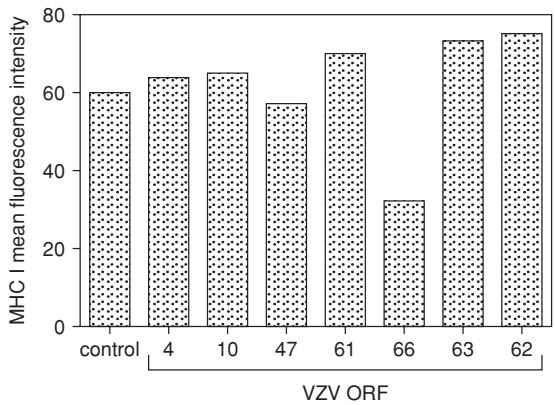

Fig. 39.2. Analysis of MHC I downregulation in cells transfected with plasmids expressing VZV proteins. HFF were transiently transfected with plasmids expressing VZV ORF4, ORF10, ORF47, ORF61, ORF62, ORF63, ORF66, or a parental control plasmid (control). 48 hr post-transfection cell preparations were stained for MHC I. Data is shown as the mean fluorescence intensity of specific cell surface MHC I staining.

In addition, VZV does not contain any identifiable homologues to the gene products of the other herpesviruses known to alter cell-surface MHC class I expression. Nonetheless, experiments using PAA to inhibit viral DNA replication suggest that an immediate-early or early VZV gene product(s) is involved in the down-modulation of cell-surface MHC class I molecules, whereas late genes were not required. VZV encodes several genes which have been reported to be expressed under immediate-early conditions (Kinchington and Cohen, 2000). To test whether these immediate-early proteins might play a role in MHC class I downregulation, HFs were transfected with the plasmids expressing ORF62, ORF63, ORF4 and ORF61. In addition, plasmids encoding the early gene products ORF10, ORF47 and ORF66 were also tested. After 48 hours, transfected cells were stained for cell-surface MHC class I expression and the mean fluorescence intensity of cell-surface MHC class I staining was determined for each transfected population of HFs by flow cytometry. Compared to the parental control plasmid, those expressing ORFs 4, 10, 47, 61, 62 and 63 did not significantly alter cell-surface MHC class I expression. In contrast, the mean fluorescence intensity of cell-surface MHC class I staining was significantly decreased on cultures transfected with the ORF66 expressing plasmid (Fig. 39.2). This is the first identification of a VZV immunomodulatory gene product that alters cell-surface MHC class I expression. Other VZV genes are also likely to encode the ability to downregulate MHC class I as another herpesvirus, HCMV, encodes no fewer than four viral genes which function to down-regulate MHC class I (Fruh *et al.*, 1999).

VZV encoded inhibition of IFN γ-mediated up-regulation of cell-surface MHC class II

Studies of adaptive immunity to VZV reveal the importance of CD4+ restricted T-cell responses during primary infection and in the maintenance of latency. Therefore, it is likely that VZV has evolved strategies for modulating the expression of MHC class II, as well as MHC class I expression. VZV specific CD4+ T-cells that are elicited during primary infection are predominantly of the Th1 type (Bergen *et al.*, 1991; Zhang *et al.*, 1994) and function to produce high levels of IFN-γ which potentiates the clonal expansion of VZV specific T-cells (Arvin *et al.*, 1986a,b; Jenkins *et al.*,1998; Wallace, *et al.*, 1994). This cytokine is also essential for the up-regulation of MHC class II on cell types that usually do not constitutively express this immune molecule. Although the classical CTL response is mediated by CD8+ T-cells that recognize viral peptides in association with MHC class I molecules, VZV specific CTLs can also exhibit MHC class II (CD4+) restricted killing of infected target cells (Cooper *et al.*, 1988; Diaz *et al.*, 1989; Hayward *et al.*, 1986, 1989; Hickling *et al.*, 1987; Sharp *et al.*, 1992). Peripheral T-cell populations contain high frequencies of CD4+ T-cells that mediate cytotoxicity against VZV-infected targets, as is also observed in HSV immunity (Arvin *et al.*, 1991; Zarling *et al.*, 1986). The potential of CD4+ T-cells to act as effective cytotoxic cells *in vivo* requires that target cells express cell-surface MHC class II molecules. These proteins are induced at sites of local inflammation, as in the case of cutaneous VZV lesions. Other herpesviruses such as human and mouse CMV have been shown to inhibit the upregulation of MHC class II expression induced by IFN-γ (Heise *et al.*, 1998; Miller *et al.*, 1998). Escape from CD4+ T-cell recognition by disrupting MHC class II expression is likely to be another VZV immune evasion strategy that has clinical significance. The block of IFNγ effects on MHC class II regulation could limit primary sensitization of T-cells to VZV peptides and delay the early amplification of VZV-specific CD4+ helper T-cells and release of cytokines at cutaneous sites of VZV replication. In vivo fibroblasts are infected by VZV and require IFNγ stimulation to up-regulate cell-surface MHC class II expression (Abendroth *et al.*, 2000). Therefore, we assessed the impact of VZV infection on IFN γ-stimulated expression of MHC class II on HFFs and the induction of genes associated with surface MHC class II expression. In these experiments, HFFs were infected with VZV and treated with 100 U/ml of IFNγ for 48 hours, beginning 12 hours post-infection.

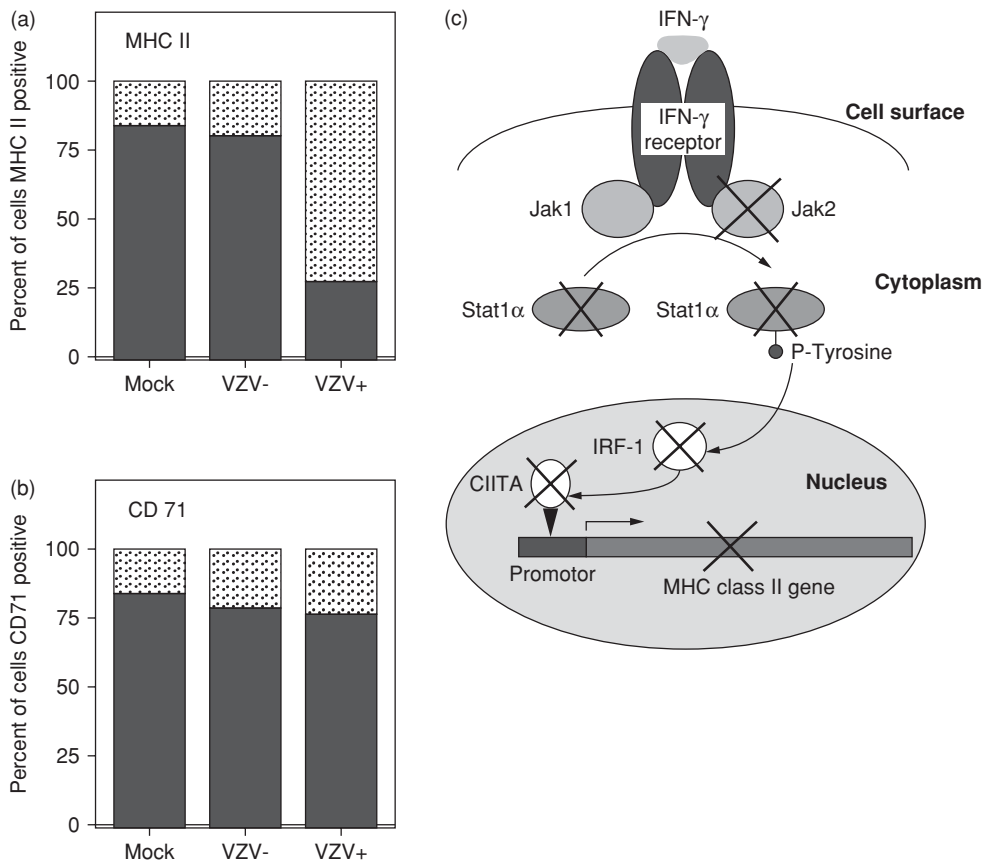

Fig. 39.3. FACS analysis of MHC class II, transferrin receptor (CD 71) and VZV proteins on VZV-infected cells treated with IFN-γ. Right panel: At 12 hours after VZV infection, HFF were treated with IFN-γ for 36 hours; cell preparations were stained with antibodies and fluorescent conjugates to MHC class II and VZV proteins or to transferrin receptor (CD71) and VZV proteins. The percentage of VZV^+ and VZV^- cell populations expressing cell surface MHC class II or CD71 is shown. Left panel: Jak/Stat signal transduction pathway, showing cellular proteins affected by VZV.

Flow cytometry analysis of VZV and cell-surface MHC class II (HLA-DR) protein expression demonstrates that IFNγ treatment induced MHC class II expression in the majority of uninfected HFs but rarely in VZV infected HFFs (Fig. 39.3). With regard to the mechanism of inhibition of IFNγ induced MHC class II gene expression, we found that MHC Class II DRα, CIITA and IRF-1 transcripts did not accumulate in VZV infected cells after treatment with IFN-γ. Stat1α and Jak2 protein synthesis was reduced compared with Jak1 and CD71, which remained unchanged (Fig. 39.3). These observations indicate that the pathway by which VZV infection alters induction of MHC class II by IFN-γ differs from the effects of HCMV and MCMV. HCMV inhibits MHC class II expression in HFFs by blocking Jak/Stat signal transduction through a specific decrease in Jak1 expression. In contrast, MCMV inhibits IFN-γ stimulated MHC class II expression in murine macrophages by a mechanism that does not involve Jak/Stat signal transduction (Heise *et al.*, 1998). Thus, among the herpesviruses, VZV, HCMV and MCMV employ different strategies to reduce MHC class II antigen presentation pathways.

The significance of the in vitro studies is substantiated by examination of skin biopsies of human varicella and herpes zoster lesions for MHC class II and VZV RNA synthesis by non-isotopic *in situ* hybridization. Cutaneous VZV lesions showed a distinct separation of VZV infected cells and cells positive for MHC class II transcripts. These experiments demonstrated that dermal and epidermal cells infected with VZV were not expressing MHC class II transcripts in vivo at early stages of lesion formation.

The persistence of VZV as a human pathogen depends upon its transmission from the cutaneous lesions that are associated with varicella, caused by primary VZV infection,

and herpes zoster, which results from reactivation of the virus from neuronal sites of latency. The ability of VZV to inhibit MHC class II expression in most infected human fibroblasts, despite exposure to high concentrations of IFNγ, provides a mechanism by which the virus can limit the consequences of immune surveillance by CD4+ T-cells. Impaired recognition of VZV-infected cells by CD4+ T-cells, which requires interaction of the T-cell receptor and viral peptides complexed with MHC class II molecules, can be predicted to allow transient viral replication in dermal and epidermal cells that is necessary for VZV transmission to susceptible individuals.

VZV interference with the NF-kB pathway

Since activation of the NF-kB pathway elicits IFN-a/b and other antiviral cytokines and proteins, herpesviruses have acquired mechanisms that inhibit this pathway. VZV interferes with NF-kB activation in cultured fibroblasts and in differentiated epidermal cells in skin xenografts in SCIDhu mice infected in vivo (Jones and Arvin, 2005). After a transient nuclear localization of the cononical NF-kB family members, p50 and p65 (Rel-A), these proteins become sequestered in the cytoplasm of VZV-infected fibroblasts. Nuclear exclusion of NF-kB proteins occurs because IkBa, which binds p50 and p65, is not degraded in VZV infected cells even though it is phosphorylated and ubiquitinated, and the 26S proteasome remains functional. VZV infection also inhibited the characteristic degradation of IkBa that is induced by exposure of fibroblasts to tumor necrosis factor alpha (TNF-α). The cytoplasmic retention of NF-kB proteins depended upon VZV replication and was in contrast to HSV-1, which induces persistent nuclear localization of p50 and p65, in a process that is required for normal HSV-1 replication. Thus, VZV has evolved a mechanism to limit host cell antiviral defenses by sequestering NF-kB proteins in the cytoplasm, a strategy that appears to be unique among the herpesviruses.

VZV infection of human dendritic cells and transmission to T-cells

The initial stage of primary VZV infection, involves the inoculation of mucosal sites with virus from respiratory droplets or cutaneous vesicle fluid from an infected individual. After inoculation, the virus remains undetected by the host immune system. (Arvin *et al., 1996*; Grose, 1981). The lymphotropism of VZV is critical for the dissemination of virus from peripheral blood mononuclear cells (PBMC) to epithelial cells, resulting in infection of the skin and the characteristic varicella rash (Arvin *et al.*, 1996). It remains unclear how VZV is transmitted from mucosal sites of inoculation to T-cells. Some T-cells may become infected in tonsillar tissues by direct transfer from infected mucosal epithelial cells (Ku *et al.*, 2004). At the same time, dendritic cells (DCs) of the respiratory mucosa may be among the first target cells to encounter VZV during primary infection and subsequently transport virus to the draining lymph nodes to enable T-cell infection as well as initiating a virus specific immune response (Jenkins *et al.*, 1999).

DCs are bone marrow-derived potent antigen presenting cells that are located in most tissues including the skin, blood, lymph and mucosal surfaces (Klagge and Schneider-Schaulies, 1999). Dendritic cells play a major role in initiating and maintaining the adaptive immune responses to pathogens such as viruses (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000). Several human viruses including human immunodeficiency virus (HIV), measles virus, influenza virus, human herpes virus 6, human cytomegalovirus and herpes simplex virus have been shown to infect human DCs (Asada *et al.*, 1999; Bender *et al.*, 1998; Fugier-Vivier *et al.*, 1997; Grosjean *et al.*, 1997; Riegler *et al.*, 2000; Salio *et al.*, 1999; Schnorr *et al.*, 1997; Warren *et al.*, 1997). There is evidence that viruses exploit DCs as transport vehicles into lymphoid tissue and ultimately contribute to the transmission of the virus in the host. Viruses are also capable of modulating or interfering with the maturation, migration and function of DCs, thereby enabling potential evasion of the immune response.

Our initial interest in DCs began with determining whether VZV could infect human dendritic cells and subsequently transfer virus to T-cells. In this study, human monocyte derived dendritic cells were inoculated with VZV strain-S and assessed by flow cytometry for VZV and dendritic cell (CD1a) antigen expression (Abendroth *et al.*, 2001b). In all human DCs inoculated with VZV, CD1a positive DCs expressing viral antigens were readily detectable (range of 15%–40%). Dendritic cells were also shown to be susceptible to VZV infection by the immunoflourescence and confocal microscopy detection of immediate-early (IE62), early (ORF29) and late (gC) gene products in CD1a+ DCs. Infectious virus was recovered from infected DCs and cell-to-cell contact was required for virus transmission to permissive fibroblasts. Significantly, VZV-infected dendritic cells were capable of transferring virus to autologous human T lymphocytes, causing productive infection of these cells. This study provides the first evidence that DC are permissive to VZV infection and that infected DCs can transfer infectious virus to T-lymphocytes.

Two possible outcomes resulting from the interaction of VZV with DCs could account for transfer of infectious

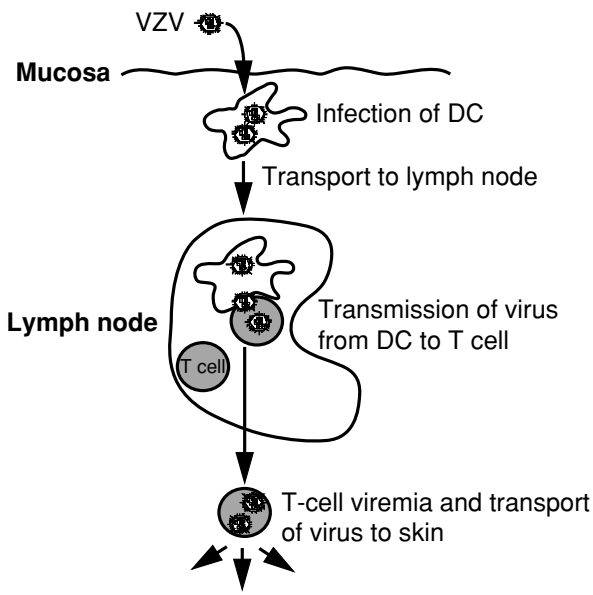

Fig. 39.4. Model of the potential role of VZV infection of dendritic cells.

virus to other cell types. Firstly, VZV may productively infect DCs and transfer new progeny virus to other cells (e.g., T-cells). Alternatively, because VZV envelope glycoproteins are known to bind mannose receptors which are found on immature DCs, the virus may be captured by DCs and internalised into trypsin resistant compartments and subsequently transmitted to other cells. Our current data clearly support the former as we were able to readily detect (by IFA and confocal microscopy) viral antigen expression from all three kinetic gene classes in sub-cellular locations consistent with those reported during productive infection of fully permissive fibroblasts (Kinchington and Cohen, 2000). This study proposed a model of VZV dissemination following primary inoculation (Fig. 39.4). In this model, upon entering the host at respiratory mucosa VZV infects DCs (Langerhans cells), which are then triggered to mobilize and migrate to the T-cell rich areas of regional lymph nodes. At this site the direct interaction of productively infected DCs with T-cells would then result in virus transmission and the productive infection of T-cells. This model, together with the potential for direct infection of T-cells by virus from VZV-infected respiratory epithelial cells are mutually compatible and consistent with a redundancy in the mechanisms by which VZV reaches T-cells. VZV pathogenesis should be facilitated by dual strategies for transfer of infectious virus into migratory T-cells. Once infection of T-cells occurs the virus should be disseminated to other sites of the body, infecting cutaneous epithelial cells with the formation of the characteristic vesicular rash of varicella. It is also possible that VZV is only captured by DCs and studies are underway to determine the mechanism of VZV entry into human DCs. Interestingly, it has been reported that HIV can either productively infect or be captured by DCs and that these two outcomes are mediated by separate pathways (Blauvelt *et al.*, 1997). Further analysis of VZV-DC interactions, including the potential to isolate infected DCs from patients undergoing primary (varicella) or recurrent (herpes zoster) infection are likely to provide additional information on these outcomes.

DCs function to present antigenic peptides on the cell surface and to stimulate T-cells. Jenkins *et al.* demonstrated that a naïve T-cell response can be induced in vitro by VZV antigenic peptides, suggesting that DCs may be involved in the initiation of the primary immune response in vivo (Jenkins *et al.*, 1999). The induction of the primary T-cell response involves not only the recognition of antigenic peptides in association with cell-surface MHC molecules, but also the interaction of co-stimulatory molecules (Marland *et al.*, 1996). Several molecules are involved in this process include CD86, CD80, CD40, CD54 and CD83 and the absence or decreased expression of these immune molecules can render a DC less capable of inducing a T-cell response (Klagge and Schneider-Schaulies, 1999). It has been postulated that interference with DC function following virus infection may enable viruses to avoid immune recognition. In this respect, measles virus and HSV have been shown to interfere with the antigen presenting capability of infected DCs by a variety of mechanisms. Measles virus can productively infect DCs and interfere with the cells ability to induce the proliferation of CD4+ naïve T-cells (Grosjean *et al.*, 1997). HSV infection of mature DCs results in a decreased T-cell stimulatory capacity and the specific degradation of the CD83 cell-surface molecule (Salio *et al.*, 1999). However, not all viruses which infect DCs interfere with DC antigen presenting function. For example, HHV-6 productively infects DCs but these cells can still function as antigen presenting cells (Asada *et al.*, 1999). As outlined above VZV has been shown to encode the ability to specifically down-modulate cell-surface MHC class I and IFNγ-induced MHC class II expression during productive infection of primary human fibroblasts (Abendroth *et al.*, 2001a, 2000). VZV-infected immature DCs showed little or no change in the level of cell-surface expression of MHC class I, MHC class II, CD40 and CD86. Current studies are in progress to determine whether VZV infection of immature DCs inhibits DC maturation given that the transition from an immature to a mature state is essential for DCs to migrate and perform their antigen presentation function. To date, several other herpesviruses

including HSV, HCMV and MCMV have been shown to inhibit the maturation of immature DCs by preventing up-regulation and expression of selective cell-surface immune molecules (Kruse *et al.*, 2000).

VZV encodes an immune evasion strategy during the productive infection of mature dendritic cells

DCs located in the periphery exist as immature cells, expressing low levels of MHC class I and MHC class II molecules and costimulatory molecules such as CD80 and CD86. Immature DCs readily take up antigen and are induced to migrate to the secondary lymphoid organs where they undergo maturation and present processed antigens to antigen specific T-lymphocytes (Steinman, 1991; Steinman *et al.*, 1997; Banchereau and Steinman, 1998). Maturation of DCs results in the down-regulation of antigen uptake and processing properties and the upregulation of MHC class I and MHC class II molecules, increased surface expression of costimulatory molecules CD80, CD86, CD40 and the maturation molecule CD83 and upregulation of adhesion molecules such as ICAM-1 (CD54) (Young and Steinman,1990; Young *et al.*, 1992; Zhou *et al.*, 1992; Zhou and Tedder, 1995; Cella *et al.*, 1997; Banchereau and Steinman, 1998; Steinman, 1999; Lechmann *et al.*, 2002). The ability of mature DCs to efficiently activate naïve T-lymphocytes which subsequently eliminate virus-infected cells has been attributed to their expression of these specific cell-surface immune molecules (Bhardwaj, 1997).

DCs should be an ideal target for viruses seeking to evade or delay the immune response by disrupting their function (Bhardwaj, 1997). In this respect, viruses including HSV-1 (Klagge and Schneider-Schaulies, 1999), HCMV (Raftery *et al.*, 2001), human herpesvirus 6 (HHV-6) (Kakimoto *et al.*, 2002), measles virus (Fugier-Vivier *et al.*, 1997; Grosjean *et al.*, 1997; Schnorr *et al.*, 1997), HIV (Blauvelt *et al.*, 1997) and lymphocytic choriomeningitis virus (LCMV) (Sevilla *et al.*, 2000) have been shown to interfere with the immune function of infected DCs by a variety of mechanisms. However, not all viruses which infect DCs interfere with DC antigen-presenting function. For example, influenza virus productively infects DCs, but these cells can still function as antigen-presenting cells (Bhardwaj *et al.*, 1994).

Given the pivotal role mature DCs play in the induction of successful anti-viral immune responses, we investigated whether VZV could infect mature DCs and interfere with their immune function. In this study, we demonstrated by flow cytometry, immunofluorescence and infectious center assays that VZV can productively infect human mature DCs and produce infectious virus (Morrow *et al.*, 2003). Following the assessment of the cell-surface expression of MHC class I, MHC class II, CD80, CD83 and CD86 by flow cytometry we found that with the exception of MHC class II, all of these molecules were down-regulated on VZV infected mature Dcs. These observations indicate that the mechanism by which VZV alters immune molecule expression on mature DCs appears novel, since the pattern of immune molecule alteration differs from that of other viruses which infect and alter mature DCs. In comparison, HSV-1 infection of mature DCs results in the down-regulation only of CD83 (Kruse *et al.*, 2000) and HSV-2 infection causes down-regulation of MHC class I, MHC class II, CD40, CD80 and CD86 on murine DCs (Jones *et al.*, 2003). It should be noted, however, that the source of cells and/or the DC maturation stimuli may have a significant bearing on the expression of immune molecules and subsequent DC function following virus infection. In this respect, HCMV has been shown to down-regulate MHC class I and MHC class II on monocyte derived DCs induced to mature with LPS or TNF-α, yet $CD34^+$ bone marrow-derived DCs induced to mature with CD40 display a down-regulation of MHC class I, MHC (Hertel *et al.*, 2003) class II, CD80, CD83, CD86 and CCR7 (E.S. Mocarski, personal communication, July 2002). Thus, among the human herpesviruses studied to date, there appear to be multiple strategies to interfere with DC immune molecule expression and the present study provides evidence that VZV has done likewise.

The most distinctive functional characteristic of mature DCs is their ability to stimulate T-cells (Banchereau and Steinman, 1998; Cella *et al.*, 1997; Steinman, 1999). Therefore, we assessed the functional consequences of the selective immune molecule alteration observed on VZV infected mature DCs. We found that VZV infection of mature DCs significantly reduced their ability to stimulate the proliferation of allogeneic T-lymphocytes. The identity of the VZV gene or genes involved in the downregulation of immune molecules and reduced T cell stimulation capacity during productive infection of mature DCs is yet to be determined. To date, ORF66 is the only identified VZV immunomodulatory gene product (Abendroth *et al.*, 2001b). Additional studies assessing ORF66 together with other viral ORFs are a focus of current experiments aimed at identifying the VZV genes responsible for the inhibition of mature DC function.

Thus, current evidence indicates that VZV is DC-tropic, and can target two distinct aspects of DC function represented by immature and mature DCs. This ability to infect both DC types confers upon the virus the potential to both increase virus dissemination in the host as well as evade the immune response.

The host immune response

Immunity during primary VZV infection

Innate immune responses are presumed to mediate the initial control of primary VZV infection. Interferon-α (IFN-α) inhibits VZV replication and is released by PBMC from naive donors exposed to VZV antigens in vitro. Early clinical trials demonstrated that the severity of varicella was reduced when immunocompromised children with varicella were given exogenous IFN-α. Our recent analysis of VZV infection of human skin xenografts in the SCIDhu mouse model of VZV pathogenesis demonstrated a dramatic increase in IFN-α production, accompanied by nuclear translocation of pStat protein within uninfected epidermal cells that surrounded skin cells actively infected with VZV (Ku *et al.*, 2004). When animals were given antibody to IFN-α/β receptor, VZV titers increased tenfold and cytopathic changes in the skin were much more extensive. Innate antiviral immunity is also mediated by natural killer (NK) cells. NK cells from naive individuals have the capacity to lyze VZV-infected targets in vitro; non-specific cytotoxicity is mediated by CD16+ T-cells and is enhanced by IL-2.

Adaptive T-cell responses, manifest as an initial burst in VZV-specific T cells, appear to be critical for resolving primary VZV infection (Fig. 39.5). Primary VZV infection is associated with an incubation period of 10–21 days before skin lesions appear. T-cell recognition of VZV is not detected in individuals tested during the incubation period, suggesting that viral mechanisms for evading immune surveillance are very effective during the initial phase of primary VZV infection (Arvin, 1999). When acquisition of VZV-specific T-cell immunity is delayed, life-threatening dissemination of the virus occurs, as described in patients with congenital T-cell immunodeficiency diseases, malignancy or immunosuppression and in newborn infants. In our early studies of antiviral immunity in immunocompromised children with varicella and malignancy, only one (7.7%) of 13 immunocompromised patients had a detectable VZV-specific T-cell proliferation response within three days after the onset of varicella compared with 19 (42%) of 45 healthy subjects ($P<0.05$) (Arvin *et al.*, 1986a). Diminished VZV-specific cellular immunity was associated with persistent VZV viremia, continued formation of new skin lesions and viral dissemination to lungs, liver and other organs. Children with HIV may develop chronic varicella, which also suggests the significance of antiviral T cell-mediated immunity in the host response to VZV. In contrast, VZV IgG and IgM antibody titers measured within the first three days, did not correlate with the severity of varicella in healthy or immunodeficient children (Arvin *et al.*, 1986a,b) and varicella appears to be uncomplicated in patients with agammaglobulinemia.

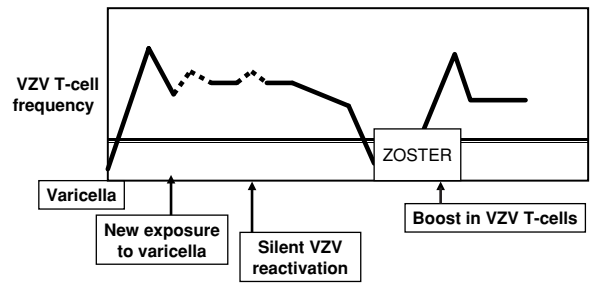

Fig. 39.5. Schema of the T-cell response to VZV.

In the healthy host with acute varicella, VZV-specific T-cell proliferation responses were detected in 12%, 31% and 47% of subjects tested at one, two and three days after the onset of rash, respectively (Arvin *et al.*, 1986a). A higher VZV-specific T-cell proliferation response, measured as the mean stimulation index (SI) at three days, was associated with fewer varicella lesions (Arvin *et al.*, 1986a). The mean VZV SI was 7.5±10.43 SD in those who had fewer than 100 lesions/m^2 when new lesion formation had stopped, compared with 1.4 ± 1.85 SD for those with >400 lesions/m^2 ($P < 0.05$). In these early experiments, activated CD4+ and CD8+ T-cells were present in peripheral blood by flow cytometry before T-cell proliferation to VZV was detected. Primary VZV infection also elicits virus-specific CD4+ T-cells that produce IL-2 and IFN-γ, which are considered Th1 cytokines. In contrast, very few VZV specific CD4+ T-cells release IL-4, a cytokine made by the Th2 subset of CD4+ T-cells (Zhang *et al.*, 1994). As expected, based on the CD4+ T cell cytokine profiles, IL-2 and IFN-γ, as well as IFN-α concentrations are increased in sera from healthy individuals with varicella (Wallace *et al.*, 1994; Arvin *et al.*, 1986a,b). Like IFN-α, IFN-γ has antiviral activity against VZV. IFN-γ, which enhances clonal expansion of antiviral T-cells, was diminished in adults with primary VZV infection compared to children. This difference may help to explain the severe cases of varicella observed in healthy adults.

Information about the VZV proteins that are recognized during the initial T cell response to primary infection is limited. Clonal expansion of T cells that recognize glycoproteins, gE and gH, and the IE62 major tegument/regulatory protein has been documented (Arvin *et al.*, 1986b). The pattern of VZV proteins that were recognized by early virus-specific CD4+ T-cells from individuals with primary VZV was variable; gE was the predominant target in 67% of healthy individuals, gH in 71% and IE62 protein in 57% of subjects. Regardless

of the predominant protein target, varicella was uncomplicated in all cases. As noted, up-regulation of class II expression permits lysis of infected cells by CD4+ cytotoxic T-cells and IFN-γ also enhances class I restricted cytotoxicity mediated by CD8+ T-cells. Although sub-populations of effector cells were not identified, high frequencies of CTL that lyze targets expressing VZV gE and IE62 protein were documented in healthy individuals with acute varicella (Diaz *et al.*, 1989).

VZV immunity during latency

VZV-specific IgG antibodies and CD4+ and CD8+ T-cells persist for decades after primary VZV infection (Arvin, 2001). VZV-specific IgG antibodies are directed against many VZV polypeptides and have functional capacities that include viral neutralization and antibody-dependent cytotoxicity (ADCC) against VZV infected targets. Most healty immune individuals also have serum antibodies to VZV.

Immune adults have VZV-specific memory T cells in peripheral blood with frequencies of approximately 1:40 000 PBMC. Glycoproteins, gE, gB, gC and gH, and the IE62 protein were recognized by T-cells in proliferation and cytokine release assays (Arvin *et al.*, 1986a). CD4+ T-cell clones specific for epitopes of gE, gB, gH or gI were derived from VZV immune donors and some clones specific for gB or gI had dual helper and cyototoxic functions. Using synthetic peptides to stimulate T-cells confirmed that regions of IE62, gB, gE and gI functioned as T-cell epitopes. In addition, VZV specific memory T-cells have been recovered from lymphoid tissue. As was reported during primary VZV infection, cytokine profiles of memory CD4+ T-cells showed that up to 85% of proliferating cells produced IFN-γ whereas only 10% released IL-4 (Hayward *et al.*, 1989). Intradermal inoculation of inactivated VZV antigens elicits delayed hypersensitivity reactions in immune children and adults (Kamiya *et al., 1977*).

MHC class I or class II restricted cytotoxic T-cells that recognize VZV proteins persist for many years after primary VZV infection (Diaz *et al.*, 1989; Hayward *et al.*, 1986; Sharp *et al.*, 1992). Memory CD4+ CTL are detected with secondary VZV stimulation and using autologous lymphoblastoid cells infected with VZV or vaccinia recombinants that express VZV proteins as targets, and CD8+ CTL can be detected withVZV-infected fibroblast targets, that express only MHC class I antigen, or with highly purified preparations of CD8+ T-cells tested against lymphoblastoid cells expressing VZV proteins. Viral protein targets that have been documented include gC, gE, gI and IE62 and IE63 proteins (Sharp *et al.*, 1992). Memory CTL specific for the IE62 protein were found at frequencies of 1:105 000 ± 85 000 SD in VZV immune subjects tested > 20 years after varicella. Mean frequencies of anti-VZV gE CTL were 1:121 000 ± 86 000 SD. Using these methods, mean frequencies of CD4+ CTLs that recognized IE62 protein were1:108 000 and 1:74 000 in the CD8+ population, which was not significantly different. VZV gE specific CD4+ CTL frequencies were 1:119 000 compared to 1:31 000 in the CD8+ population (NS).

Persistence of VZV-specific memory

The host response to VZV must protect against new exposures to varicella and against VZV reactivation from latently infected cells in the sensory ganglia (Fig. 39.5). At the same time, these exogenous and endogenous exposures to infectious VZV, constitute mechanisms by which VZV immunity may be boosted. VZV immune adults and children tested after household exposures to varicella have been found to develop increases in VZV IgG, IgM and IgA antibodies and in VZV-specific T-cell proliferation as well as enhanced delayed hypersensitivity to VZV skin test antigens (Gershon *et al.*, 1984).

Preserving VZV immunity is also important for maintaining VZV latency. VZV may undergo periodic reactivations that are asymptomatic or result in mild, undiagnosed episodes of zoster. Endogenous reactivation of VZV provides a second potential mechanism for sustaining VZV immunity over all or most of the lifetime of the host. Whereas other herpesviruses are readilty shown to reactivate at mucosal surfaces asymptomatically, VZV shedding at mucosal sites has not been documented. Evidence that subclinical VZV reactivation occurs has been suggested in studies monitoring VZV immune markers over time in healthy adults with no recent exposure to varicella. Intermittent increases in these responses may represent responses to asymptomatic VZV. The re-appearance of VZV IgM antibodies has been considered such a marker (Gershon et al. 1984). More directly, we found that 19% of bone marrow transplant patients tested during the period of highest risk for herpes zoster in these patients had subclinical cell-associated VZV viremia as detected by PCR. VZV DNA has also been detected in PBMC from some elderly individuals (Devlin *et al.* 1992). A relationship between VZV reactivation and preservation of cell-mediated immunity was suggested by the fact that many bone marrow transplant recipients had reconstitution of VZV-specific T-cell responses during the first year after transplantation, without having had any exposures to varicella or clinical signs of zoster. Recovery of VZV-specific CTL function was also observed in 50% of BMT patients who had remained asymptomatic.

Immunity during VZV reactivation

The clinical experience demonstrates a direct correlation between the increased risk of zoster and diseases or treatments that interfere with T cell function. Patients with HIV infection who have progressive depletion of CD4+ T-cells also have a high incidence of zoster. The age-related increase in the risk of zoster in healthy elderly people is also well documented and several studies have shown that VZV-specific T cell responses decline with age. In contrast, VZV IgG antibodies do not decline in high risk populations and lower titers do not predict a higher risk of VZV reactivation, causing zoster (Webster *et al.*, 1989). Elderly peole have decreases in frequencies of memory T-cells that recognize VZV in proliferation and CTL assays and delayed hypersensitivity responses to VZV skin testing are absent or diminished. In limiting dilution proliferation assays, older individuals showed reduced frequencies of CD4+ T-cells, primarily within the IFN-γ producing Th1 subset, as compared to IL-4 positive CD4+ T-cells.

In the healthy individual with zoster, the equilibrium between the virus and the host is re-established rapidly. Viral replication at skin sites is usually controlled within 1 or 2 weeks. Transfer of infectious virus into T-cells from sites of virus replication in dorsal root ganglia or skin appears to be unusual, since skin lesions are rarely found beyond the affected dermatome. In immunocompromised patients, severe, prolonged suppression of cellular immunity is associated with the highest risk of herpes zoster and of life-threatening dissemination of the virus, resulting from cell-associated VZV viremia (Arvin, 1999). Among bone marrow transplant recipients, memory CD8+ T-cells are generated after in vitro stimulation with VZV antigen in numbers comparable to those of healthy immune individuals whereas CD4+ CTL function is difficult to detect during the period after transplantation when these patients are at high risk for VZV recurrences. The observation that the risk of herpes zoster increases when patients with HIV infection have low CD4+ T-cell numbers is consistent with a role for CD4+ as well as CD8+ T-cells in the restriction of viral replication in vivo. Immunocompromised patients often have scattered cutaneous lesions indicating the occurrence of viremia during zoster. Under these circumstances, VZV may enter DC in the involved skin area and reach T-cells in regional lymph nodes, or it may be transferred into migratory T-cells, causing viremia as a result of the limitations of the host response.

Herpes zoster is followed by increased VZV IgG, IgM and IgA antibodies in healthy and immunocompromised patients. The humoral immune response is directed against a broad range of VZV proteins, including early, structural/regulatory proteins, viral enzymes and glycoproteins. Nevertheless, the contribution of humoral immunity to resolving VZV reactivation is not certain. Some individuals who have early responses with high titers of VZV IgG and IgM antibodies have severe herpes zoster whereas those with mild clinical disease may have limited increases in VZV antibody titers. Viral replication at local cutaneous sites was not inhibited when VZV immune globulin was given to immunocompromised individuals with herpes zoster before treatment with antiviral drugs was possible. However, there was some evidence that a diminished humoral immune response early in the clinical course was associated with a higher risk of viremia and viral dissemination in immunocompromised patients.

Healthy adults with herpes zoster develop a marked increase in VZV-specific T-cell responses, as shown by comparing VZV T-cell proliferation within one week after onset with responses tested from two to four weeks later. IFN-α is detected in vesicle fluid from the cutaneous zoster lesions, increasing as the lesions resolve in healthy subjects (Stevens *et al.*, 1975). Herpes zoster in immunocompromised patients is associated with delayed boosting of VZV T-cell responses. In one study of patients with malignancy and zoster, the mean SI was 1.8 ± 0.85 SD during the first week and had increased only to 5.7 ± 3.03 SD by 2 to 4 weeks. IFN-α concentrations were also low in zoster lesions in these high-risk patients and IFN-α treatment accelerated the resolution of zoster in such patients (Merigan *et al.*, 1978).

The introduction of the live attenuated varicella vaccine has introduced new considerations about the persistence of memory immunity to VZV, which are discussed in Chapter 37. Such vaccines may prove to be useful clinically as a strategy for reversing the waning VZV T cell immunity that occurs with age. In a recent study, we provided a "proof of concept" for this approach by demonstrating that inactivated varicella vaccine given to hematopoeitic cell transplant recipients resulted in early reconstitution of VZV-specific CD4 T cell proliferation and a reduced risk of symptomatic episodes of zoster (Hata *et al.*, 2002). Since the live attenuated varicella vaccine can boost VZV T cell immunity in healthy elderly adults, the successful outcome of studies to examine its effectiveness for preventing or modifying the severity of zoster suggests that VZV-specific T cells help to block the progression of VZV reactivation from latency to clinically symptomatic infection (Oxman *et al.*, 2005).

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40

VSV: persistence in the population

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Like other herpes viruses, varicella zoster virus (VZV) causes disease due to the primary infection (varicella) and due to reactivation (herpes zoster). However, VZV differs from other herpes viruses in causing primary and reactivation infections that are easily recognized clinical diseases, even by the lay public. Because of this, the epidemiology of varicella and herpes zoster has been well described from clinically recognized disease (incidence, severe disease outcomes and deaths) with seroprevalence data providing additional information on the epidemiology of varicella especially in populations where varicella disease history may not be available.

Varicella occurs worldwide with ongoing endemic transmission in areas where populations are sufficiently large to support such transmission. However the epidemiology of varicella varies between temperate and tropical climates (Lee, 1998). Universal childhood vaccination programs have changed the epidemiology of varicella in countries implementing such programs with significant declines in disease. Most experience has been gained in the United States where a varicella vaccination program was initiated in 1995. Herpes zoster infections also occur throughout the world although the epidemiology of herpes zoster is less well described globally. Because the incidence of herpes zoster increases dramatically with age, countries with lower life expectancies may have lower health burdens due to this disease. A vaccine for prevention of herpes zoster and post-herpetic neuralgia was licensed in the USA in May, 2006 (Oxman *et al.*, 2005). This chapter reviews pre- and post-vaccine epidemiology of varicella and herpes zoster.

Varicella: prevaccine epidemiology

Transmission

Varicella is a highly infectious viral disease caused by the varicella zoster virus (VZV) that results from exposure to cases of varicella or herpes zoster. Herpes zoster cases represent a method for regular exposure and reintroduction of VZV into communities that otherwise may not be large enough to sustain endemic transmission of the virus. Herpes zoster is less transmissible than varicella. This may relate both to the limited number of lesions and to modes of transmission; the infection is most commonly localized and is not thought to involve the respiratory tract. Varicella can be transmitted by respiratory droplets, from skin lesions, by direct contact or possibly by aerosolization of virus from skin lesions and also, presumably from lesions in the mouth (enanthem). Studying transmission of varicella has been challenging due to the strong cell association of the virus. VZV has been consistently difficult to culture from the throat but easy to culture from skin lesions. Finally, infection of a pregnant woman in the first 2 trimesters of pregnancy may result in transplacental transmission of VZV resulting in a severe congenital infection in the fetus or newborn known as congenital (fetal) varicella syndrome (Enders *et al.*, 1994).

Varicella transmission has been described from household studies where secondary attack rates among susceptible children following household exposure has ranged from 61% to 100% (Asano *et al.*, 1977; Hope-Simpson, 1952; Ross, 1962). The highest estimates come from small groups of seronegative children involved in postexposure vaccine effectiveness studies in household settings. The lowest estimate is from observations in a general practice in England where children <15 years with a negative disease history were followed after exposure to a primary household case (Hope-Simpson, 1952). Hope-Simpson described transmissibility of varicella to be lower than measles (76%) but higher than mumps (32%). In a large study in the United States of the effect of gamma globulin on modifying varicella infections, Ross described a secondary attack rate of 87% among untreated children aged 6 months to 12 years with a negative disease history (Ross, 1962).

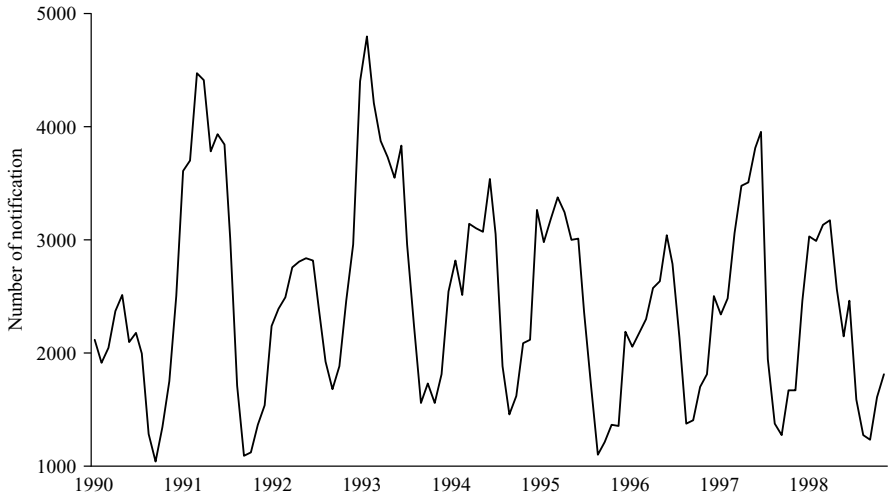

Fig. 40.1. Notification of varicella in Scotland, 4-week periods, 1990–1998.

Compared with households, transmission is lower in community settings; the relatively few published estimates available come from varicella outbreak investigations that have included vaccinated as well as unvaccinated children. In these situations, due to continuing exposure, outbreaks in child care centers may ultimately achieve a cumulative attack rate among history and vaccine negative children as high as 80%–88% (Galil *et al.*, 2002b; Izurieta *et al.*, 1997) though, depending on the size of the child care center, this may take weeks or months of transmission. In elementary schools, cumulative attack rates among susceptible children tend to be lower (30%–54%) perhaps due to different mixing patterns resulting in lower risk of exposure (Dworkin *et al.*, 2002). Yorke and London, in analyses of 30 to 35 years of monthly reported measles and varicella cases from New York City and Baltimore, suggested that varicella is only 35%–65% as infectious as measles in the community (Yorke and London, 1973).

Herpes zoster represents a method for reintroduction of VZV into the community in the absence of epidemic varicella. Patients with herpes zoster are less contagious than cases of varicella because their infection is localized and it is not thought to involve the respiratory tract. However, some studies have identified VZV antigen (by PCR) in throat samples of healthy persons with localized herpes zoster. There are few published studies on the transmissibility of herpes zoster probably because of the limited opportunities for exposure of susceptible persons to herpes zoster cases which occur predominantly, among older adults. However, Seiler reported that 11 (15.5%) of 71 susceptible children <15 years of age exposed to herpes zoster in household settings developed varicella (Seiler, 1949). This proportion increased to 17% if infants <1 year of age, who may be protected by maternally acquired antibodies, were excluded from the calculation. Young children with herpes zoster may be more contagious. In a daycare setting, a 3-year-old boy with herpes zoster transmitted in the secondary generation to ~30% of susceptible children in the day care he attended and to both his susceptible siblings (Reigle and Cooperstock, 1985). The child was reported to "lift his shirt repeatedly to scratch or to show everyone his lesions." As varicella disease declines in countries implementing universal childhood vaccination programs, transmission from herpes zoster may become more apparent.

Periodicity and seasonality

Worldwide, varicella is an endemic disease that exhibits a marked seasonal pattern in temperate climates and most temperate climates where this has been studied (Bramley and Jones 2000; Degeun *et al.*, 1998; Lee, 1998; Seward *et al.*, 2002; Tobias *et al.*, 1998) (Fig. 40.1). The exception is Singapore where seasonality has not been described from surveillance data (Ooi *et al.*, 1992). In temperate and tropical climates, the peak disease incidence is most commonly reported in the cooler, drier months during winter or spring. Periodicity with interepidemic cycles of 2–5 years is described from many countries (Bramley and Jones, 2000; Degeun *et al.*, 1998; Seward *et al.*, 2000; Tobias *et al.*, 1998) while a time period as long as 15 years between major epidemics has been described from Singapore (Ooi *et al.*, 1992).

Congenital (fetal) varicella syndrome

This condition was first described in 1947 (Laforet and Lynch, 1947). It is characterized by cicatricial skin lesions and neurologic, eye and skeletal anomalies which may include limb paresis, hypoplasia of upper or lower extremities, chorioretinitis, cataracts and cortical atrophy. Based on a large, multicenter study where 1373 women with varicella and 366 with herpes zoster during pregnancy were prospectively studied, the risk of congenital (fetal) varicella syndrome was found to be approximately 0.4% if varicella infection was acquired in the first 12 weeks of pregnancy and 2.0% from 13–20 weeks (Enders *et al.*, 1994). Maternal zoster was not associated with fetal abnormalities. Based on this study, Enders estimated that the rate of fetal varicella syndrome may be 1.6 per 100 000 births. Enders and Miller used these findings to estimate the number of cases of congenital varicella syndrome that may occur every year by applying a varicella age-specific incidence of 2/1000 population among women of child-bearing age to the number of births per year, taking into account the risk of infection occurring in the first 20 weeks of pregnancy. They calculated that, in the prevaccine era, there may have been 44 cases of congenital varicella syndrome every year in the United States, 8 in England and Wales and 9 in Germany (Enders and Miller, 2000). Incidence is likely to be higher in countries where VZV susceptibility is higher among adults though data are lacking.

Incidence and seroprevalence

Age and climate

Varicella is a highly contagious disease that occurs worldwide. The epidemiology of varicella varies between temperate and tropical climates. Though incompletely understood, these differences may relate to agent, host, environmental or a combination of these factors (Garnett *et al.*, 1993; Lolekha *et al.*, 2001; Mandal *et al.*, 1998; Seward *et al.*, 2000). Because climatic differences (as distinguished from an "island" effect reflecting reduced risk of exposure) are not observed for other highly contagious diseases such as measles, and VZV is known to be heat labile, a partial explanation may be that heat diminishes the ability of the virus to survive in the environment and thereby decreases transmission. In most temperate climates, >90% of persons are infected by 15 years of age with the highest incidence of disease occurring among children <10 years of age (Seward *et al.*, 2000; Wharton, 1996). In tropical climates, cases are acquired at older ages with a higher proportion of cases and higher susceptibility among adults (Lee, 1998).

Varicella incidence data has been described mainly from developed countries where data are collected from notifiable disease reporting, surveys or studies based on medical record encounters. Prior to the national varicella vaccination program in the United States, total annual varicella incidence measured from national household survey data, averaged over a decade, was 15.0–16.0 cases per 1000 population (Guess *et al.*, 1986; Seward *et al.*, 1998). In other temperate climates, reported incidence using different methods of data collection have been generally lower than US rates varying from <5 to ~13.0 cases per 1000 total population (Boelle and Hanslik, 2002; Bramley and Jones, 2000; Choo *et al.*, 1995; Fairley and Miller, 1996). Higher rates, especially from a single year of data collection, may reflect an epidemic disease year (Chant *et al.*, 1998) (Table 40.1).

Infectiousness of diseases is reflected in age-specific incidence. As stated by Hope-Simpson following studies of the infectiousness of measles, varicella and mumps in the household "the more infectious the disease, the younger is the age at which an attack is likely to be received" (Hope-Simpson, 1952). Studies from earlier in the twentieth century in the US showed the highest varicella age-specific incidence was among children aged 6–7 years, in the first 2 years of school (Fales, 1928). To understand age-specific disease patterns, single years of age or small age groups should be studied; grouping ages 5–14 years, for example, may mask considerable variations in incidence within this broad age span. Age-specific incidence among children 5–9 years from US national data in the 1970s and 1980s was ~90/1000 children (Guess *et al.*, 1986; Wharton, 1996). However, by the 1990s, varicella was being acquired at earlier ages. Before introduction of varicella vaccine in the United States in 1995, varicella age-specific incidence had shifted to earlier ages with preschool aged children (1–4 years) having higher age specific incidence than the 5–9-year-old age group (Seward, 1998) (Table 40.1). These data are consistent with findings in many countries including France, Italy, England and Wales, Scotland and Slovenia where the highest age-specific incidence in the 1990s has been reported among preschool-aged children <5 years (Bramley and Jones, 2000; Degeun *et al.*, 1998; Fornaro *et al.*, 1999; Ross and Fleming, 2000) with peak incidence in some studies as young as 1 or 2 years (Socan *et al.*, 2001, Yawn *et al.*, 1997). These changes in epidemiology are thought to reflect earlier exposure through attendance in child care (Wharton 1996; Yawn *et al.* 1997; Ross and Fleming, 2000)

Varicella seroprevalence data reflect age-specific disease incidence and in countries where incidence data are not available, VZV age-specific seroprevalence provide an excellent method for understanding the epidemiology of

Table 40.1. Varicella age-specific and total incidence, selected studies

Country, author, publication year	Study method	Years data collection	Age (years)						Annual total varicella incidence/1000 population
			<1	1–4	5–9	10–14	15–19	20+	
United States (Guess *et al.*, 1986)	National survey	1970–1978	33.8	82.1	90.3	17.5	2.9	0.3	16.0
United States (Seward *et al.*, 1998)	National survey	1990–1994	56.7	100.4	91.0	19.5	4.9	1.5	15.0
France (Boelle *et al.*, 2002)	Sentinel physician reporting	1990–1999	49.8	121.2	36.0		3.4		12.6
United States (Choo *et al.*, 1995)	Health maintenance organization records	July 1990– June 1991 July 1991– June 1992	52.3		41.3	14.0	6.1	1.8	7.6, 12.0
England and Wales (Fairley and Miller, 1996)	Sentinel physician reporting	1989–1990	40.0		17.3		3.1 (15–44 years)		6.3
Scotland (Bramley and Jones, 2000)	Sentinel physician reporting	1989–1990	14.5–35.0	31.4–63.4	16.4–27.8		2.3–6.1 (15–24 years)		5.4–8.9 (European standardized rate)
Australia (Chant *et al.*, 1998)	Local survey	1995	110		130	60	40		33.9

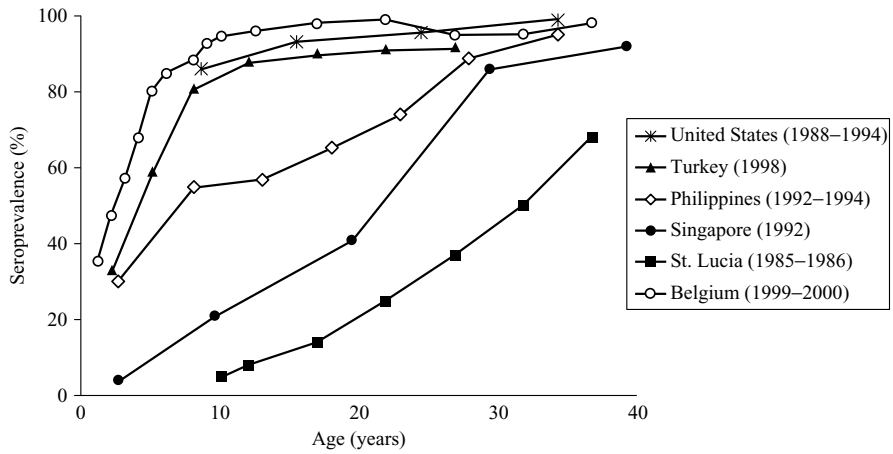

Fig. 40.2. VZV Seroprevalence by age in eight countries.

the disease. Comparing results from serosurveys should take into account improvements in sensitivity and specificity of laboratory methods over time. In addition, these studies represent a snapshot of the seroprevalence status in a community; seroprevalence will be higher if samples are obtained after a varicella epidemic. In temperate climates, >90% of adolescents or young adults are VZV seropositive (Fig. 40.2) (Kanra *et al.*, 2002; Kilgore *et al.*, 2003; Seward *et al.*, 2000; Thiry *et al.*, 2002). Slightly lower seroprevalence rates in adolescents (10–15 years) (82%) are reported from Italy whereas a study in South Africa reported a seroprevalence of 81% among adults 20–29 years (Gabutti *et al.*, 2001; Schoub *et al.*, 1985). In tropical climates, serological studies show higher susceptibility among adults reflecting a higher mean age of infection than in temperate climates (Fig. 40.2). In such countries, seroprevalence among adolescents or young adults has varied widely from less than 20% in St. Lucia (an island community in the West Indies) to >90% in tropical areas in Brazil and urban Calcutta, India (Barzaga *et al.*, 1994; Garnett *et al.*, 1993; Lee, 1998; Mandal *et al.*, 1998; Ooi *et al.*, 1992; Reis *et al.*, 2003).

Urban/rural (risk of exposure)

Urban/rural differences in varicella epidemiology are likely to be due to varying risks of exposure due to differences in population density. Analysis of surveillance data collected in the 1920s in Maryland, US, between 1913 and 1917, showed an earlier mean age of varicella infection among urban (6.7 years) compared with rural children (8.6 years) (Fales, 1928). Studies exploring reasons why the epidemiology of varicella differs between temperate and tropical climates have examined the effect of climate and population density on VZV seroprevalence. In Thailand, significantly lower age-adjusted VZV seroprevalence was found in the warmer than in the cooler regions and in the warmer regions only, the age-specific seroprevalence was significantly higher in the urban population than the rural population (Lolekha *et al.*, 2001). Urban/rural differences have also been described in Calcutta and neighboring rural areas in India. Mandel found that 96% of urban young adults aged 17–25 years were immune compared with 42% of similarly aged rural adults (Mandal *et al.*, 1998). In Eritrea, VZV seroprevalence among an isolated adult population was 44% compared with other adult groups in the same areas where immunity ranged from 91%–96% (Ghebrekidan *et al.*, 1999). Although these data suggest that the higher susceptibility in the tropics may reflect reduced exposure, this is not the only explanation for the differences described. In St. Lucia, seroprevalence for VZV was lower than for mumps, a far less infectious disease and in Singapore, which is densely populated, VZV seroprevalence among young adults has consistently been lower than in countries with temperate climates (Garnett *et al.*, 1993; Ooi *et al.*, 1992).

Other factors: sex, race, number of siblings in the household and child care

Most studies show no differences in seroprevalence by sex. In the United States, national data and studies conducted among military recruits have described differences in varicella susceptibility by race with higher susceptibility among African Americans compared with whites (Jerant *et al.*, 1998; Kilgore *et al.*, 2003). In the national data, these differences narrow with increasing age and are not apparent after approximately age 40. Some studies have shown differences in seroprevalence according to number of siblings

in the household during childhood (Jerant *et al.*, 1998; Ryan *et al.*, 2003). Because of its high incidence in young children, varicella is one of the most common communicable diseases in child care centers and attendance in child care or preschools provides the opportunity for exposure to the varicella zoster virus (VZV) at younger ages. Children attending day care have higher incidence or prevalence of varicella and an increased risk of exposure to varicella with increasing size of the center (Hurwitz *et al.*, 1991; Seward *et al.*, 2000).

Hospitalization and deaths

Though often considered a benign childhood disease, varicella may result in serious complications and death. This health burden assumes greater importance as other infectious causes of morbidity and mortality such as polio, measles and *H. influenzae* are controlled through vaccination (Rawson *et al.*, 2001). Assessing the severe health burden due to varicella in terms of economic and societal costs is important for vaccine policy decision making and to monitor the impact of vaccination programs. Data on population-based varicella mortality, case fatality and hospitalizations are rarely available from developing countries or countries with tropical climates where a higher proportion of cases occur among adults. Additionally, methodological differences in data collection as well as issues such as access to health care should be considered when comparing studies, especially across countries. Finally, calculating case fatality rates and risks of hospitalization for varicella cases is dependent on having accurate incidence data available.

In the United States, in the 5–8 years before licensure of varicella vaccine, varicella resulted in an average of 10 632 hospitalizations and 100 deaths per year (Galil *et al.*, 2002a; Meyer *et al.*, 2000); two-thirds of the hospitalizations and about half the deaths occurred in children. Although varicella is a more severe infection in immunocompromised persons, the majority of severe morbidity and mortality in developed countries occurs among healthy persons. In France (1990–1997), 70% of all varicella deaths and in the United States (1990–1994), 89% of varicella deaths among children and 75% of varicella deaths among adults occurred in persons without underlying high risk medical conditions (including HIV/AIDS, leukemia and other malignancies, other forms of blood dyscrasia and immune deficiencies) (Boelle and Hanslik, 2002; Meyer *et al.*, 2000).

In the US, between 1970 and 1994, before the use of varicella vaccine, crude varicella mortality rates (examining varicella as the underlying cause of death) declined from 0.7 per million population in 1973 to 0.2 in 1986 and then increased to average 0.4 per million population from 1990–1994 (Meyer *et al.*, 2000). Similar crude mortality rates are reported from Australia (0.3/million), France (0.35/million), England and Wales (0.5/million), Scotland (0.5/million) and Singapore (0–0.8/million) (Boelle and Hanslik, 2002; Bramley and Jones, 2000; Chant *et al.*, 1998; Fairley and Miller, 1996; Lam *et al.*, 1993; Rawson *et al.*, 2001). Reflecting the high incidence of varicella in children, varicella mortality rates are highest among children especially those <1 year of age (1.1–3.6 deaths per million population) and lowest among adults (0.2–0.3 deaths per million population).

The risk of dying from varicella is measured by the case fatality rate (CFR). In the US, data from 1970–1994 show higher case fatality rates in infants <1 year and adults ≥20 years of age compared with children 1–9 years (Meyer *et al.*, 2000). Even though CFRs among adults declined substantially from the 1970s to the 1990s, during 1990–1994, adults still had a 27 times higher risk of dying from varicella (CFR 21.3 per 100 000 cases) than children 1–4 years (0.8/100 000 cases). Similar CFRs are reported from France from 1990–97 (CFR 1.0/100 000 for children <15 years and 22.8 for persons ≥15 years) and England and Wales from 1988–1992 for persons <45 years (0.7 for 0–4 years, 1.4 for 5–14 years and 20 for persons 15–44 years of age) (Boelle and Hanslik, 2002; Fairley and Miller, 1996). Though extremely high CFRs (471 and 535) are reported for adults ≥65 years in England and Wales, and France respectively, in this age group, misclassification of varicella with herpes zoster is more likely to occur, as documented in US hospitalization and mortality data (Choo *et al.*, 1995; Galil *et al.*, 2002c).

Population-based mortality data are lacking from developing countries and from countries with tropical climates. Because of the older age of infection and case severity among adults, tropical countries may experience greater morbidity and mortality from varicella and its complications, including congenital varicella syndrome. The eradication of smallpox afforded an opportunity to study varicella in developing countries in the 1970s in more detail than has been possible since. When smallpox was still endemic, varicella was the rash illness most commonly confused with smallpox (Jezek *et al.*, 1978a). For several years after smallpox eradication, heightened surveillance for febrile rash illnesses was conducted. In India, 862 155 varicella cases and 433 varicella deaths were reported from January to December 1976 for a CFR of 5.2 per 10 000 reported cases, 50 times higher than in developed countries (Jezek *et al.*, 1978b). Reasons for the higher case fatality may include incomplete ascertainment of cases (reporting bias for more severe cases) compared with deaths, varicella epidemiology in India, and access to, and quality of, medical care. For 400 deaths where both age and sex were recorded, 80% occurred among adults >15 years and 71%

were male, reflecting varicella epidemiology in tropical climates and perhaps a reporting bias and/or medical access differences for adults and for males compared with females (Jezek *et al.*, 1978a).

Varicella hospitalizations represent severe morbidity or health burden due to varicella and its consequences, and the infection control burden due to varicella infections in hospitals. As pointed out by Wharton, "ascertaining the reason for hospitalization from hospital discharge diagnoses, in the absence of additional information from the clinical record, is difficult due to lack of standard procedures for ordering hospital discharge diagnoses" (Wharton, 1996). Some studies have validated hospital discharge codes (Choo *et al.*, 1995). Thus, when comparing studies of hospitalizations, especially across countries, it is important to consider differences in methods that may result in higher or lower estimates including how hospitalizations were identified (from discharge codes or from reviewing medical records), whether the primary discharge or all discharge codes were searched for varicella, the validity of discharge codes and how hospital admission practices, billing and coding may change over time.

Estimates of annual hospitalizations for varicella in the US during the 1970s and 1980s ranged from approximately 4000 to 9000 depending on the dates of the study, the population studied, and study methods (Guess *et al.*, 1986; Wharton, 1996). More recent estimates indicate annual hospitalizations attributable to varicella of 10 632 per year (Galil *et al.*, 2002a) and almost 15 000 per year if all varicella hospitalizations are included (Ratner, 2002). Reflecting the range of these estimates, crude varicella hospitalization rates from the US, France, Australia, Scotland, and England and Wales have varied from approximately 2–6 per 100 000 population with more recent estimates attempting to describe attributable hospitalizations in the US varying from 3.1–4.1 per 100 000 population (Boelle and Hanslik, 2002; Bramley and Jones, 2000; Chant *et al.*, 1998, Fairley and Miller, 1996; Galil *et al.*, 2002a, Ratner, 2002). The majority of varicella hospitalizations occur among children (56%–67%) reflecting the fact that 90% of varicella cases occur among this age group.

For all ages combined, overall rates of hospital admission per 1000 varicella cases have ranged from 2.2 to 4.7 in national studies in the US and France (Boelle and Hanslik, 2002; Galil *et al.*, 2002a; Ratner, 2002). The highest rate from France included principal and associated varicella hospitalizations. In the 1990s, among children <13 with varicella, a hospitalization rate of 5.5 per 1000 cases was reported from Minnesota (Yawn *et al.*, 1997). The risk of hospitalization varies by age. The pattern of age-specific risks for hospitalizations is similar to that of age-specific case fatality rates with infants and adults having higher risks of hospitalization than young children. Except in France where the risk for hospitalization is higher in infants than in adults, studies consistently report the highest risk of hospitalization in adults, an increased risk in infants and the lowest risk in children 1–4 years or 5–9 years of age (Boelle and Hanslik, 2002; Fairley and Miller, 1996; Galil *et al.*, 2002a; Guess *et al.*, 1986; Wharton, 1996).

Studies of varicella hospitalizations in developing countries are sparse and population-based hospitalization rates are commonly not available for comparison with data from developed countries. However, similar to reported varicella mortality described above (Jezek *et al.*, 1978a), studies of varicella hospitalizations in tropical climates also describe a high proportion of hospitalized cases among adults and also males, which may, in part, reflect hospital admission practices (Seward *et al.*, 2000).

The HIV epidemic may also be expected to influence the epidemiology of varicella. In countries with high HIV prevalence, varicella may cause more severe morbidity and mortality however there are few population-based data examining these issues. A retrospective review of all children admitted to the only isolation facility in Durban, South Africa from 1986–1996, showed a decline in all disease mortality of 86% over the study period mainly attributed to a decline in measles deaths. However, between 1994 and 1996, 15% of varicella admissions and 75% of varicella deaths occurred in HIV co-infected children (Jeena *et al.*, 1998).

Varicella epidemiology: post-vaccine era

A vaccination program is implemented in order to reduce, eliminate or eradicate disease. Because current strains of live attenuated varicella vaccine are neurotropic and are capable of reactivating to cause herpes zoster, albeit at lower rates than wild virus, eradication of VZV infections is not possible with currently licensed vaccines. The goal of a universal varicella vaccination program is to greatly reduce or eliminate varicella disease, especially severe disease.

The United States was the first country to implement a national varicella vaccination program in 1995 and active surveillance for varicella was established in sentinel sites to monitor impact of the vaccination program. By 2000, in these communities, vaccine coverage among children 19–35 months had reached 74%–84% and reported total varicella cases and hospitalizations had declined 71%–84% (Seward *et al.*, 2002) (Fig. 40.3, Table 40.2). Although incidence declined to the greatest extent (83%–90%) among children 1 to 4 years, incidence declined in all age groups including infants and adults indicating herd immunity effects. In the combined surveillance

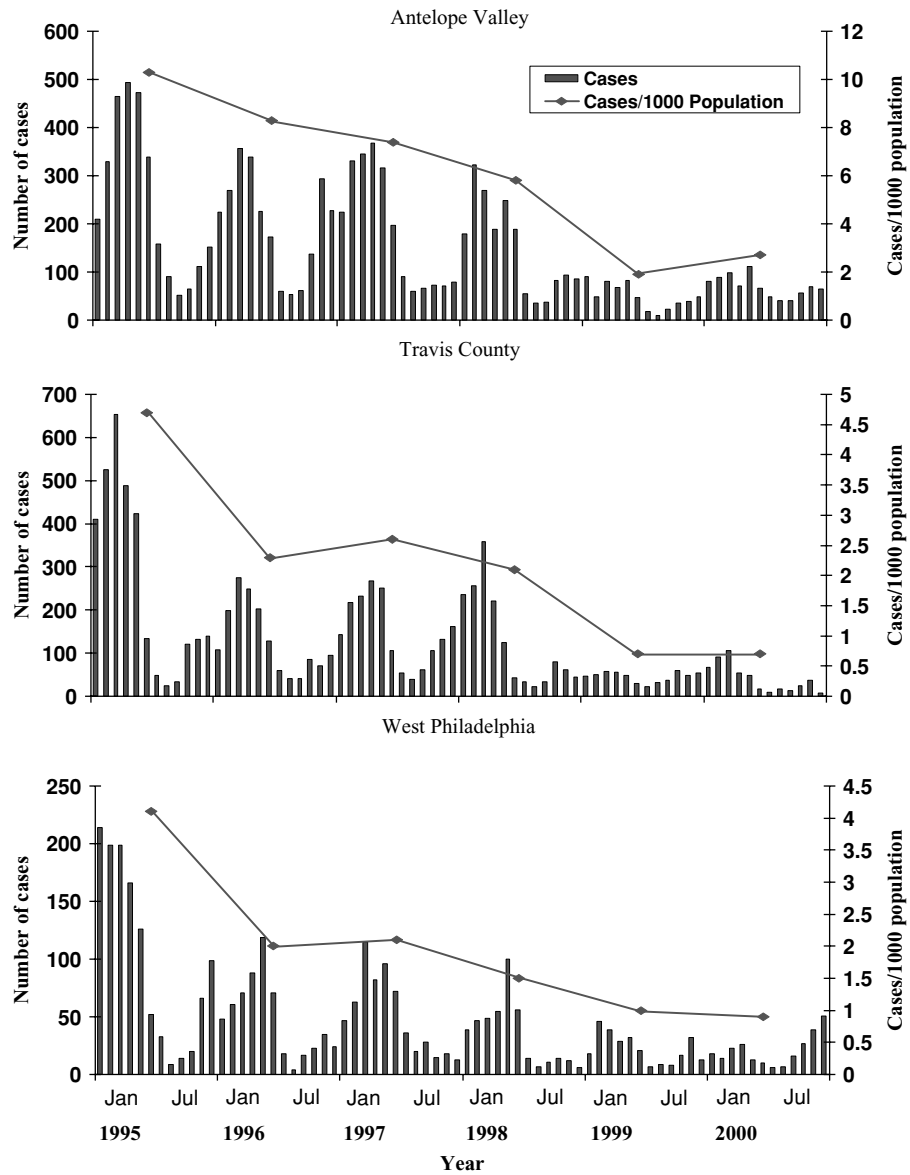

Fig. 40.3. Reported varicella cases by month and annual rates of reported cases per 1000 population in three surveillance areas, 1995–2000.

areas, varicella-related hospitalizations declined from a range of 2.7–4.2 per 100 000 population from 1995 to 1998 to 0.6 and 1.5 per 100 000 population in 1999 and 2000, respectively.

In the USA, a decline in varicella incidence has also been documented from passive surveillance systems. In four states with adequate (≥5% of expected cases) and consistent rates of reporting to the national notifiable disease surveillance system, compared with the average incidence for 1990–1994, reductions in varicella incidence in 2001 ranged from 67% to 82% with vaccine coverage among children 19–35 months ranging from 57%-84% (Centers for Disease Control & Prevention, 2003). Additionally, implementation of a varicella serological screening and vaccination program in Navy military recruits has been followed by an 80% decline in cases in this population (Ryan *et al.*, 2003). At the national level, significant declines in varicella mortality, and in varicella-related

Table 40.2. Percent reduction of reported varicella cases in 2000 compared to 1995, three surveillance areas, United States

Age	Antelope Valley, California	Travis County, Texas	West Philadelphia, Pennsylvania
	%	%	%
<1	69	81	68
1–4	83	90	83
5–9	63	77	77
10–14	65	75	80
15–19	85	83	81
≥20	66	64	68
Total	71	84	79

Note: From Seward, J. F, Watson, B. M., Peterson, C. L. *et al.* (2002).

hospitalizations and their attendant costs have been also been documented in the United States (Nguyen *et al.*, 2005; Zhou *et al.*, 2005; Davis *et al.*, 2004). Among persons less than 50 years of age, varicella deaths declined 74% or more from the immediate pre-vaccine era (1990–1994) to 1999 to 2001 and deaths in children 1 to 9 years of age declined approximately 90% (Nguyen *et al.*, 2005). For hospitalizations, from the prevaccination period to 2002, hospitalizations due to varicella declined by 88% and ambulatory visits declined 59% (Zhou, 2005).

Routine childhood vaccination programs are the most effective strategy for interrupting disease transmission and reducing varicella mortality and morbidity in both temperate and tropical climates. Achieving high vaccination coverage among children will provide the additional benefits of herd immunity with protection of susceptible adults, infants and other persons at high risk for severe varicella disease who are not eligible for vaccination. For programs that achieve high vaccination coverage, the most dramatic effect will be a marked reduction in varicella cases, severe complications and deaths in the population as is now occurring in the United States (Seward *et al.*, 2002; CDC unpublished data, 2003). Among the remaining greatly reduced number of varicella cases, a higher proportion is expected to occur among older persons, as was seen following the introduction of vaccines against measles, mumps and rubella. The shift in the proportion of cases to older persons will be minimized by catch-up vaccination of older children and adolescents. In contrast, vaccination programs targeting only adolescents and adults will have little impact on the epidemiology of varicella in temperate climates. In the US such a targeted program, assuming that all susceptible adults could be vaccinated, would be expected to result in only a 5%–10% decline in cases, a 33% decline in hospitalizations and a 50% decline in deaths. In tropical countries, providing vaccine for susceptible adolescents and adults in addition to infants and children may be more important because a higher proportion of adults are likely to be susceptible.

Although some other countries, including Uruguay, Qatar, Australia, Canada, and Germany (2005) have implemented universal childhood varicella vaccination programs, no data are yet available on the impact of these programs. Some countries are waiting for the availability of a combination MMRV vaccine (licenced in the USA in 2005) before implementing a universal vaccination program and others are considering adolescent vaccination programs. Varicella vaccines are now widely available through the private sector throughout the world. If partial and sustained vaccine uptake occurs through the private sector reaching coverage levels in the range of 30%–60%, adverse epidemiological effects may occur resulting in an increase in incidence or severe morbidity in adolescents and adults similar to those predicted for selective rubella vaccine use (Vynnycky *et al.*, 2003).

A varicella vaccination program has the potential to change the epidemiology of herpes zoster as well as varicella. Therefore, surveillance for herpes zoster as well as varicella is important for monitoring the vaccination program.

Herpes zoster epidemiology

Methodological issues

Studies of herpes zoster (HZ) incidence, morbidity and mortality have used various methodologies; the most reliable data come from prospective cohort studies. Herpes zoster incidence has been described from surveillance data where there is likely to be significant under-reporting of cases, retrospective cohort studies that use medical records thus missing cases that do not seek medical attention, and surveys that may encounter non responders. Thus, differences in the method of data collection must be taken into account when comparing HZ incidence data. In addition, because HZ incidence increases dramatically with age, comparing HZ incidence rates across studies needs to take this into account by standardizing rates to a reference population. Other methodological issues arise, especially when assessing disease burden due to HZ. Herpes zoster is more common among elderly adults, an age group that is likely to have chronic medical conditions that may cause lengthy hospitalizations or death; therefore, it is important to describe the attributable health burden or mortality from HZ. Otherwise, coincidental HZ infections that occur

during hospitalization and that may be listed in hospital discharge codes and on death certificates may inflate health burden and mortality due to HZ. MacIntyre *et al.* (2003) reported that only 40% of 4718 hospitalizations that listed HZ in discharge codes had HZ as the primary diagnosis, and that these cases had significantly shorter hospitalization stay and were less likely to die (1%) than those with a secondary HZ diagnosis (6%).

Seasonality and clustering

Because HZ results from reactivation of latent VZV, the disease does not occur in epidemics and periodicity is not described. Most studies report no seasonal variations in the incidence of HZ (Brisson *et al.*, 2001; Hope-Simpson, 1965; McGregor, 1957; Ragozzino *et al.*, 1982); however several studies report seasonality with peak incidence in the summer and some authors speculate that this may be associated with ultraviolet radiation that peaks in the summer months (Glynn *et al.*, 1990; Wilson, 1986; Zak-Prelich *et al.*, 2000).

Although small clusters of HZ cases have been reported (Hope Simpson, 1965; Palmer *et al.*, 1985; Schimpff *et al.*, 1972), most authors report no clustering, or if clustering occurred they were considered to represent coincidental episodes of disease (Brisson *et al.*, 2001; Donahue *et al.*, 1995; Glynn *et al.*, 1990; Hope-Simpson, 1965; Wilson, 1986). A few authors suggest that clusters may represent a clinical manifestation of exogenous reinfection, or stimulation of endogenous VZV reactivation, (Palmer *et al.*, 1985; Schimpff *et al.*, 1972).

Secular trends

Several studies have suggested that HZ rates are increasing. In a study in the United States spanning 15 years from 1945–1959, Ragozzino *et al.* (1982) reported an increase in the annual age standardized (1970 US white population) incidence from 1.1 to 1.5 per 1000 person years. In his study in the 1990s, Donahue *et al.* (1995) reported that once the HZ incidence rates he observed were standardized to the 1970 US white population, they increased to 2.9 per 1000 person–years, more than double that reported by Ragazzino. The increase was not associated with aging of the population or with an increase in immunosuppressed individuals. In addition, from 1979 to 1997, increases in HZ incidence of 35% and 21% have been described in Canada and England, respectively (Brisson *et al.*, 2001).

Dermatomal distribution

Most studies report no laterality to the HZ rash with both the right and left sides affected equally. The rash affects mainly one dermatome with the thoracic, cervical, lumbar, and cranial dermatomes accounting for over 90% of the dermatomes affected (di Luzio Paparatti *et al.*, 1999; Guess *et al.*, 1985; Hope-Simpson, 1965; McGregor, 1957; Petursson *et al.*, 1998; Ragozzino *et al.*, 1982). Hope-Simpson noted that the HZ rash distribution correlates with the distribution of the varicella rash that follows a centripetal distribution. The rash affects mostly the trunk (thoracic 50%), and face (cranial 13%) with similar percentages for the cervical (14%) and lumbar (13%) regions, and spares the limbs. Similar findings were reported for HZ rashes in children (Guess *et al.*, 1985; Takayama *et al.*, 2000).

Incidence

Age

It is estimated that about 10%–30% of people will get HZ during their lifetime, resulting in about 300 000–900 000 cases of HZ in the US, and 260 000 cases in the UK each year (Brisson *et al.*, 2001; Schmader, 2001). The incidence of HZ varies markedly in published studies. The total population incidence of HZ, unadjusted for age differences between populations, ranges from 1.3 to 4.8 per 1000 person–years (Table 40.3) (Chidiac *et al.*, 2001; McGregor, 1957; Ragozzino *et al.*, 1982). The differences in the rates observed across studies may be due to differences in the methods used for ascertainment of cases, case definitions, determination of population at risk, health systems capturing cases, and age distribution of population studied (that may be skewed or not reflective of the overall population) rather than to true population differences. In a prospective cohort study of all patients in his general practice in Cirencester, UK from 1947–1962, Hope-Simpson reported an annual incidence rate of 3.4 per 1000 person–years (Hope-Simpson, 1965). Much lower incidence rates were found in a community-based study in the US in which cases were identified retrospectively from medical records in a central diagnosis system that included all providers in the community (Ragozzino *et al.*, 1982). The denominator included the population of Rochester, Minnesota, obtained from census data. Other studies used well defined populations for denominators such as enrollees in a health plan in Massachusetts (Donahue *et al.*, 1995), attendants at general practices in Scotland (McGregor, 1957), England, (Hope-Simpson, 1965), and Italy (di Luzio Paparatti *et al.*, 1999). The study in France (Chidiac *et al.*, 2001) was a prospective study that did not include cases from pediatricians or from residents in institutions, and used the population from the census for the denominator. Therefore, it is important to adjust rates of studies that report age-specific incidence to a standard population for comparison purposes. The

Table 40.3. Herpes zoster age-specific and total incidence, selected studies

Country, author, publication year	Study method and population	Number of cases	Years data collection	Age (years)								Annual total zoster incidence/ 1000 population
				0–9	10–19	20–39	40–49	50–59	60–69	70–79	80+	
United Kingdom (Hope-Simpson, 1965)	Prospective General practices	192	1947–1962	0.7	1.4	2.4	2.9	5.1	6.8	6.4	9.4	3.4
France (Chidiac *et al.*, 2001)	Prospective General practices and dermatologists	8103	1997–1998	2.2		2.4	5.4		9.9 (60–74 yrs)	12.8 (75+yrs)		4.8
				0–4 yrs	5–9 yrs	10–14 yrs	15–19 yrs					0–19 yrs
United States (Guess *et al.*, 1985)	Retrospective Medical records for Population	173	1960–1981	0.20	0.30	0.59	0.63		NA			0.42
Iceland (Petursson *et al.*, 1998)	Prospective General practices	121	1990–1995	0.8	1.5	2.3	1.8		NA			1.6
				Age (years)								
				0–14	15–24	25–44	45–54	55–64	65–74	75+		
United States (Ragozzino *et al.*, 1982)	Retrospective Medical Records of the Population	590	1945–1959	0.30	0.8	1.0	1.5	2.4	3.4	4.4		1.3
United States (Donahue *et al.*, 1995)	Retrospective Health maintenance organization records	1075	1990–1992	0.46	1.03	2.10	3.13	5.71	9.99	14.24		2.15
Netherlands (Opstelten *et al.*, 2002)	Retrospective Medical records in General Practices	837	1990–1995	2.1 (0–44 yrs)			3.6	5.8	6.5	9.1		3.4
England (Brisson and Edmunds, 2003)	Surveillance of General Practices	Not available	1991–2003	0.92 (0–4 yrs)	2.19 (5–14 yrs)	2.12 (15–44 yrs)		7.12 (45–64 yrs)		9.32 (65+ yrs)		3.73
Scotland (McGregor, 1957)	Prospective General Practice	81	1948–1955	1.2 (0–5 yrs)		2.2 (15–44 yrs)		8.1 (45–64 yrs)		10.4 (65+ yrs)		4.8

differences in incidence rates for two studies (Donahue *et al.*, 1995; Hope-Simpson, 1965) narrowed from 2.1 and 3.4 per 1000 person–years respectively to 3.2 and 3.3 per 1000 person–years as a result of adjusting the crude rated to the 2000 US population, highlighting the need for such adjustments before comparing rates between studies.

The majority of studies report that a small proportion of persons infected with HZ may experience a second or a third episode. Hope-Simpson (1965) reported that the rate of second or more infections in persons previously infected is similar to that of first infection in the general population with 8 (4.1%) of 192 cases reporting a second episode, and one person a third episode, resulting in a rate of 3.1 per 1000 person–years. These findings were supported by other studies with recurrence ranging from 1.7%–5.2% (Donahue *et al.*, 1995; Ragozzino *et al.*, 1982; Wilson, 1982). A much higher recurrence rate of 45% was reported in one English study in persons with a first infection occurring over the age of 45 years (Edmunds *et al.*, 2001).

All published studies report that the incidence of HZ increases with increasing age (Brisson *et al.*, 2001; Chidiac *et al.*, 2001; Donahue *et al.*, 1995; McGregor, 1957; Hope-Simpson, 1965; Ragozzino *et al.*, 1982; Opstelten *et al.*, 2002) (Table 40.3). Most studies report an approximately 10 fold increase in risk with increasing age; Hope-Simpson reported incidence increasing from 0.74 among children less than 10 years of age to 10.1 per 1000 person–years among individuals aged 80–89 years. This marked increase in incidence with age results in up to 50% of people who live to 85 years acquiring HZ (Brisson *et al.*, 2001; Schmader, 2001).

Because children may not have acquired varicella and therefore may not yet be at risk for reactivation of VZV, the incidence of HZ also depends on varicella epidemiology. Therefore, comparison of HZ incidence rates in children need to take into account both the age distribution of children in the studies and the age-specific incidence of varicella which may vary across populations. Hope-Simpson, 40 years ago, speculated that the increase in HZ incidence in persons 0–20 years reflected the increase in the number of children infected with VZV, placing them at risk for HZ. He suggested that the incidence observed in the third decade, by which time almost everyone in temperate climates is latently infected with VZV, reflects the incidence in a maximally infected population. Studies of HZ in children report annual incidence rates ranging from 0.25 to 1.15 per 1000 population for children <10 years and from 0.43 to 1.60 for those <20 years (Chidiac *et al.*, 2001; Guess *et al.*, 1985; Hope-Simpson, 1965; Petursson *et al.*, 1998) (Table 40.3). Other studies used different age groups such as <5, and 5–14 years, or <14 years making comparison between studies difficult (Brisson *et al.*, 2001; Donahue *et al.*, 1995; McGregor, 1957; Ragozzino *et al.*, 1982). Guess *et al.* (1985) in Rochester, Minnesota, during the period 1960–1981, reported that the incidence of HZ increased with age from 0.20 cases per 1000 person–years in children less than 5 years of age to 0.63 cases per 1000 person–years in those 15 to 19 years of age. In contrast, Petursson *et al.* (1998) in Iceland, during 1990–1996, reported rates that ranged from 0.80 per 1000 person-years in children less than 5 years to 1.80 in those 15–19 years of age (Table 40.3). These four fold differences in rates of HZ in children in narrow (5 year) age groupings are more likely to signify differences in study methods including completeness of ascertainment than differences between populations (Guess *et al.*, 1985; Petursson *et al.*, 1998). Further studies to understand such differences are needed. Maternal varicella during pregnancy and varicella or variable exposure during the first few months of life are associated with HZ in childhood.

Sex

The majority of studies among all ages or among the elderly find no significant differences between males and females in crude or age-adjusted HZ incidence (Brisson *et al.*, 2001; Donahue *et al.*, 1995; Guess *et al.*, 1985; MacIntyre *et al.*, 2003; Wilson, 1986; Petursson *et al.*, 1998; Ragozzino *et al.*, 1982; Schmader *et al.*, 1995), while some studies report slight differences in rates between males and females (di Luzio Paparatti *et al.*, 1999; Hope-Simpson, 1965; McGregor, 1957), and others report higher rates among females (Chant *et al.*, 1998; Chidiac *et al.*, 2001; Cooper, 1987; Thomas and Hall, 2004). Wilson (1986) found that younger males 0–20 years had a higher incidence when compared to females of the same age. One study in the US found that females had a higher crude hospitalization rate of 18.5 compared to 13.4 among males; however, once age adjusted, the differences disappeared (Lin and Hadler, 2000). Although a study from Australia reported a 2.2 times higher HZ mortality rate among females (0.092 per 100 000) compared to males (0.043); these rates were not age adjusted and may be affected by the longer life expectancy in females (Chant *et al.*, 1998).

Race

The effect of race on incidence of HZ has been studied mainly by Schmader in the United States. In several studies among elderly racially diverse populations in North Carolina, blacks had a significantly lower lifetime occurrence and annual incidence of HZ than whites. In a community-based study among persons >64 years old in North Carolina, Schmader *et al.* (1995) reported that 16.1% of elderly white persons reported HZ compared with only 4.5% of elderly blacks ($P < 0.0001$). Even after controlling for age,

cancer, and demographic factors, blacks were 4 times less likely than whites to have experienced HZ (adjusted odds ratio (aOR), 0.25, 95% Confidence Interval (CI), 0.18–0.35). In a follow-up prospective study in the same community between 1989 and 1994, Schmader *et al.* (1998) reported that after controlling for age, sex, education, cancer, other chronic diseases, hospitalization, activities of daily living, self-rated health, depression, and cigarette smoking, black individuals were a little over a third as likely to develop HZ than were white individuals (aOR, 0.37; 95% CI, 0.26, 0.53; $P = 0.0001$). Hypothesized reasons for the lower risk of HZ among elderly blacks include age at onset of varicella, racial differences in VZV immunity, and lifetime exposures to varicella (Schmader, 2000). Thomas and Hall (2004), in a recent analysis of Morbidity Statistics from General Practice (MSGP) studies in the UK, have corroborated these findings with black adults having less than half the risk of HZ after adjusting for age, sex and country of birth (risk ratio (RR), 0.46, 95% CI, 0.21–0.97). However, in this analysis, the protective effect for blacks was not associated with household exposure to children.

Stress

Few studies have examined the effect of psychological stress on risk of HZ although there are case reports of HZ occurring after a stressful event. Schmader has studied psychological stress as a risk factor for HZ (Schmader *et al.* (1990). In a community-based case control study where HZ cases were matched for age, sex, and race, he reported that psychologically stressful life events were risk factors for HZ. Cases experienced negative life events significantly more often than controls 2 months before (OR, 2.60, 95% CI, 1.13, 6.27), 3 months before (OR, 2.64, 95% CI, 1.20, 6.04), or 6 months before HZ onset (OR, 2.00, 95% CI, 1.04, 3.93). Cases were more likely to perceive recent events as stressful compared to controls. In a prospective study where recall bias was not an issue, stressful life events increased the risk of HZ but the result was borderline for statistical significance (aOR, 1.38; 95% CI, 0.96–1.97) (Schmader *et al.*, 1998).

Age of varicella infection

Some researchers have speculated that the lower risk of HZ among elderly black adults may be due to later age of acquisition of varicella. Thomas and Hall (2004) attempted to address this question for adults born in tropical climates where age of varicella infections are later than in temperate climates. Persons born in countries described as having stronger evidence of late-onset varicella (the Caribbean, Central America, India, Pakistan, Sri Lanka, Bangladesh, Singapore and Malaysia), had a lower risk of HZ compared with adults born in the UK after adjusting for age and sex (RR, 0.56, 95% CI 0.28–1.12, $P = 0.072$) although these differences were not statistically significant (Thomas and Hall, 2004). Additional studies are needed to verify that this is due to age at varicella infection and not other factors related to country of birth or migration.

Exposure to varicella

Hope-Simpson first speculated that immunity to VZV may be maintained by periodic internal reactivation of VZV, external boosting of immunity through exposures to varicella or HZ or both. A number of studies have either directly or indirectly examined the role of contacts with varicella in both immunocompromised and healthy populations. Garnett and Grenfell (1992) examined weekly reported data in a time series analysis and reported no association between varicella and HZ although they reported some correlation with the annual data for some age groups. Three other studies suggest that contact with varicella cases appear to lower the risk of HZ in adults (Solomon *et al.*, 1998; Terada *et al.*, 1995; Thomas *et al.*, 2002). However, the level of exposure reported to affect herpes zoster in the above studies is unlikely to occur for most of the population. Two analyses reported that exposure to children was associated with a lower HZ risk (Brisson *et al.*, 2001; Thomas and Hall, 2004). A detailed discussion of this topic is presented in the "Impact of vaccination" section below.

Immunocompromising states

Herpes zoster is more common among individuals with depressed cell-mediated immunity from immunosuppressive disorders including cancer, especially hematological cancers (certain leukemias and lymphomas), HIV infection and transplants, and from immunosuppressive medications. A higher proportion of persons with HZ are immunosuppressed compared to the general population (Ragozzino *et al.*, 1982; Guess *et al.*, 1985; Rusthoven, 1994; Donahue *et al.*, 1995; Lin and Hadler, 2001). Guinee *et al.* (1985) conducted a retrospective cohort study of HZ from six cancer centers over a 3-year period. The cumulative incidence of HZ in the 717 patients identified with Hodgkin's disease was 9.5% after 1 year, 16.6% after 2 years and 20.6% after 3 years. To further study this issue, in a large prospective study in Canada of HZ in cancer patients >15 years of age with a minimum of 5 years of follow-up, Rusthoven *et al.* (1988) found that the cumulative incidence rate of HZ 5 years after diagnosis was highest in hematological malignancies (14% in Hodgkin's disease, 10% in leukemia and 5% in non-Hodgkin's lymphoma) compared with solid tumors (breast 2%, lung 2%, and gynecological malignancies 1%). These differences were not due to age; in fact the median age of HZ patients with hematological malignancies was younger (51 years) than patients

with solid tumors (59 years, $P < 0.005$). The finding of the highest risk of HZ in patients with Hodgkin's disease has been reported by other investigators in both pediatric and adult populations (Rusthoven, 1994; Schmader, 2000). Persons with cancers of any kind have an increased HZ risk with the administration of chemotherapy and radiation (Rusthoven, 1994). Feld *et al.* (1980) reported that 13 (8.1%) of patients with small-cell anaplastic carcinoma of the lung treated with chemotherapy and radiation developed HZ.

Persons with HIV infection also have a higher risk of incident and recurrent HZ although the risks are more comparable to solid tumors than hematological malignancies. Studies from the US, the Netherlands, Australia and Uganda that have compared rates of HZ in HIV-positive and HIV-negative persons have described 12–17 times higher risk among HIV-positive persons (Buchbinder *et al.*, 1992; McNulty *et al.*, 1997; Morgan *et al.*, 2001; Veenstra *et al.*, 1996). Incidence rates among HIV-positive persons varied in the above studies from 29.4 cases to 51.5 per 1000 person–years. Recurrence rates also varied according to the length of follow up, ranging from 10% to 25.6% (Morgan *et al.*, 2001; Veenstra *et al.*, 1996). In the Ugandan study, the incidence of HZ increased with increasing time since seroconversion to HIV + status from 7.6% at 2 years to 24.0% at 6 years. Differences in rates across studies in HIV+ populations may relate to differences in immune status and viral load because of differences in availability of effective treatment.

Persons who undergo allogeneic or autologous bone marrow transplant (BMT) experience a high risk of HZ, soon after the procedure. A variety of studies in different countries have reported this risk to range from 17%–52% (Locksley *et al.*, 1985; Nader *et al.*, 1995; Rusthoven, 1994; Schuchter *et al.*, 1989; Tzeng et al., 1995). The risk is highest in the months immediately following the procedure; the majority of HZ cases occur within a year of transplantation. Schuchter reported that 28% of 151 autologus BMT patients developed HZ after the procedure, and that 91% of the cases occurred within the first year; these findings were supported by another study in which 82% of the HZ cases occurred within the first year following BMT (Tzeng *et al.*, 1995).

Complications

Herpes zoster may lead to complications such as persistent pain (postherpetic neuralgia), bacterial infection of the lesions, pneumonia, encephalitis, and hemorrhagic complications. A number of risk factors have been found to be associated with complications, the most important of which are older age and immunosuppression. Persons >64 years have about eight times the risk of complications compared to those <25 years (Galil *et al.*, 1997). Other factors include trigeminal distribution of the HZ rash, involvement of more than one dermatome (di Luzio Paparatti *et al.*, 1999; Galil *et al.*, 1997), severe pain, rash or prodromal symptoms at HZ presentation (Choo *et al.*, 1997; Dworkin *et al.*, 2001; Nagasako *et al.*, 2002; Whitley *et al.*, 1998), persisting abnormal sensations in the affected dermatome (Decroix *et al.*, 2000) and scarring, presumed to be a consequence of rash severity (Battcock *et al.*, 1990; Bowsher, 1999). Patients who presented with severe or incapacitating pain and a large number of lesions were 18 times less likely to achieve resolution of both acute neuritis and HZ-associated pain (Whitley *et al.*, 1999).

Postherpetic neuralgia (PHN), a chronic pain syndrome, is the most common complication of HZ (Johnson, 2002; Lojeski and Stevens, 2000; Ragozzino *et al.*, 1982; Stankus *et al.*, 2000). The variability in the intensity and duration of the pain has made it complicated for researchers to agree on a standard definition making it difficult to compare results across studies. Studies have variously defined onset of PHN at rash onset or rash resolution. Study end points vary from 1, 3, 6 and 12 months after rash onset or resolution. At one month past rash onset, reported proportions of persons with PHN range from 6.5% to 45% (Haanpaa *et al.*, 2000; Opstelten *et al.*, 2002), at 3 months, the range is from 7.2% to 25% (Haanpaa *et al.*, 2000; Helgason *et al.*, 2000), and at 12 months, 3.4% to 10% (Bowsher, 1999; Helgason *et al.*, 2000). The varying proportions in PHN at the different time periods may be affected by several factors including age and percent of immunosuppressed in the population under study.

The risk of PHN increases with increasing age although the magnitude of the effect varies across studies. PHN is more common among adults older than 50 years (Bowsher, 1999; Decroix *et al.*, 2000; di Luzio Paparatti *et al.*, 1999; Dworkin *et al.*, 2001). It is estimated that about 27–68% of HZ cases over 60 years of age experience PHN (Dworkin *et al.*, 2001; Kurokawa *et al.*, 2002). In an Iceland study, older age was a significant and independent predictor of PHN; persons 55–74 years were 4.2 times more likely to develop PHN one month after rash onset compared to those younger than 55 years; while those over 75 years of age were 10.7 more likely to develop PHN (Opstelten *et al.*, 2002). An even stronger association with older age was reported in a U.S. study where age ≥50 years compared to age <50 years was associated with a 14.7-fold higher prevalence of PHN at 1 month and a 27.4-fold higher prevalence at 2 months after developing HZ (Choo *et al.*, 1997). Finally, in Singapore, Goh and Khoo (1997) reported that 20% of patients

older than 50 years suffered from PHN compared to 7% of persons less than 30 years of age. Some of the difference in magnitude of the effect of older age on PHN may be due to the fact that baseline comparison groups used in the above studies differed. Furthermore, some studies controlled for other risk factors (Choo *et al.*, 1997; Opstelten *et al.*, 2002) while others did not (Goh and Khoo, 1997).

Herpes zoster hospitalizations and complications in healthy children are rare. Guess *et al.* (1985) found low morbidity among children compared to adults. Among 173 HZ cases in person <20 years of age, there were no occurrences of post-herpetic neuralgia or other late complications and only 2 (1%) were hospitalized. Furthermore, in Iceland, none of the 112 (118 episodes) cases <20 years of age developed moderate or severe pain during the acute illness or postherpetic neuralgia (95% CI, 0 to 0.03) (Petursson *et al.*, 1998).

Hospitalizations

Fewer population-based data are available on HZ hospitalizations and no studies report hospitalization rates that are adjusted for age and high risk conditions. As with HZ incidence, the hospitalization rates increase with increasing age and high rates are seen in persons with suppressed immune systems. In the US, statewide hospital discharge data from Connecticut from 1986–1995 showed an annual crude HZ hospitalization rate of 16.1 per 100 000 person years (Lin and Hadler, 2000). A much lower crude incidence rate was observed in Northern California (2.1 per 100 000 health maintenance organization members) (Coplan *et al.*, 2001), and an intermediate rate of 4.4 per 100 000 was reported in England for 1995 to 1996 (Brisson and Edmunds, 2003). However, because the Connecticut study included all HZ diagnoses rather than HZ as the primary discharge diagnosis, hospitalization rates may have been over-estimated by including individuals with coincidental HZ (Lin and Hadler, 2000). In this study, 31.4% of all hospitalized HZ cases and the majority of HZ hospitalizations among person <50 years of age (61%) had at least one underlying condition that increased the risk or severity of HZ; the majority of these were immunocompromising conditions (82% malignancies, and 8% HIV infection). In contrast, only 8% of the HZ hospitalized cases in England had at least one underlying condition; malignancies accounted for 87% and HIV infection for 6% of the conditions listed (Brisson and Edmunds, 2003). Finally, 67% of the hospitalized cases in the Connecticut study were 64 years or older, while only 55% of those in the California study were 60 years or older emphasizing the importance of age adjustment when comparing studies.

The rates of HZ hospitalizations increase sharply among the elderly (Chant *et al.*, 1998; Coplan *et al.*, 2001; Lin and Hadler, 2000). In one US study, HZ hospitalization rates increased from 21.3 per 100 000 populations among individuals <30 years of age to 1604.5 per 100 000 among those 85 years of age or older. The steepest increase in rates occurred among those over 64 years of age (Lin and Hadler, 2000). Similar patterns are seen in other studies and other countries with hospitalization rates increasing after age 50 or 60 years though rates vary across studies (Brisson *et al.*, 2001; Brisson and Edmunds, 2003; MacIntyre *et al.*, 2003). In Australia, HZ hospitalization remained stable at about 25 per 100 000 populations among persons <60 years of age, then increased from about 50 per 100 000 for the 60–64 age groups to over 300 for the persons 85 years and older (MacIntyre *et al.*, 2003). In England and Canada, HZ hospitalization rates increased from 2 and 1 per 100 000 population in children aged <5 years to 148 and 86 in adults older than 64 years of age, respectively (Brisson *et al.*, 2001).

Some studies of HZ hospitalization did not report rates. In Singapore, between 1993 and 1994, HZ accounted for 3% of total hospitalizations with a mean age of 50 years (range 23 months to 88 years); 58% of HZ hospitalizations were older than 50 years of age (Oh *et al.* 1997). In contrast, HZ hospitalization in Australia accounted for 0.08% of all hospitalizations between 1998 and 1999 with a mean age of 69 years and 53% were older than 50 years (MacIntyre *et al.*, 2003). Chant *et al.* (1998) reported that more women in Australia than men were hospitalized for HZ (ratio of women to men was 1.4:–1.6:1), while Oh *et al.* (1997) reported no sex differences in hospitalization in Singapore.

Deaths

Studies on HZ mortality are few and study methods may not be comparable. As with incidence, hospitalizations, and complications, HZ mortality is more common among the elderly and those with suppressed immune systems. Schmader (2000) surmized based on clinical experience and the absence of HZ-related deaths in cohort studies, that HZ mortality appears to be an infrequent event at least among healthy persons. In Australia during 1971–1993, Chant *et al.* (1998) reported that 92% of HZ deaths per year occurred among persons older than 65 years. The average crude death rate for the study period was 0.068 per 100 000 population and was 10 times higher for the elderly >65 years (mortality rate 0.60). Females had more than double the rate of death compared to males however these crude mortality rates are not age adjusted (Chant *et al.*, 1998). In Lin's study in the US, 5.3% of all HZ-related hospitalizations resulted in death and about 52% of those who

died had at least one underlying high risk condition, including malignancies; leukemia, and HIV. The risk of death was higher for those with underlying high risk conditions (8.7%) than for those without any high risk conditions (3.7%). In a more recent study in Australia, 4% of 4718 hospitalized for HZ died; however, death was much less common in the group with primary HZ hospitalization (1%) compared to the group with HZ as the secondary diagnosis for hospitalization (6%) (MacIntyre *et al.*, 2003). Finally, in a study in England and Wales from 1993 to 2000, the overall HZ mortality rate was 0.094 per 100 000 person–years however, the number of HZ deaths decreased from 64 in 1993–1994 to 40 in 1999–2000 (Brisson and Edmunds, 2003). The risk of death was <0.014 per 100 000 person–years in persons aged <64 years then increased sharply to 0.566 for persons older than 64 years. Finally, similar results were observed for case fatality ranging from <2 per 100 000 HZ cases in persons <65 years of age to 61 per 100 000 HZ cases in persons older than 64 years of age (Brisson and Edmunds, 2003).

Impact of vaccination

The impact of child and adult vaccination against VZV on the incidence of HZ and PHN remains to be determined. In the US, where there are 9 years of experience with a universal varicella vaccination program, varicella incidence, hospitalizations, and mortality have declined dramatically (Seward *et al.*, 2002; Nguyen *et al.*, 2005; Zhou, 2005). In a study using data from a large Health Maintenance Organization, there was no change in the overall or age-specific incidence of HZ between 1992 and 2002 in an area with about 70% varicella vaccination coverage and a decline in varicella disease that started in 1999 (Jumaan *et al.*, 2005). Survey data from 2000 onwards from Massachusetts show an increase in herpes zoster (Yih *et al.*, 2005).

In 1965, Hope-Simpson hypothesized that the long interval observed between the infection with varicella disease, resulting in the establishment of the latent virus, and the reactivation to cause HZ may be due to internal and external boosting. The external boosting hypothesis, suggesting the need for frequent exposures to VZV to maintain immunity against reactivation of the latent virus has attracted attention, especially with the licensure of the varicella vaccine. Some researchers have speculated that a universal varicella vaccination program, with its associated decline in varicella disease, may have the unintended effect of increasing the incidence of HZ. They note that the decline in varicella disease due to the vaccination program leads to fewer opportunities for persons with a latent virus to boost their immunity through exposure to children with wild-type VZV infection.

Modeling the impact of universal varicella vaccination predicts an initial increase in the incidence of HZ that will occur within 50 years until HZ declines as vaccinated cohorts replace those with a history of varicella disease (Brisson *et al.*, 2002; Garnett and Ferguson, 1996). Although the incidence of HZ is much lower than that of varicella, some studies suggest that the health burden due to HZ is greater than that due to varicella because of the higher rates of complications, hospitalizations and deaths (Lin and Hadler, 2000; Brisson and Edmunds, 2003; Chant *et al.*, 1998; MacIntyre *et al.*, 2003). This has been raised as a serious potential concern in other developed countries considering a varicella vaccination program. However, the health burden on HZ may be overestimated. In a study in England, varicella and HZ hospitalization rates were similar at 4.5 and 4.4 per 100 000 population, respectively (Brisson and Edmunds, 2003). Furthermore, in another study in England and Canada, the reported age specific proportion of cases hospitalized and in-patient days hospitalized were only slightly higher for HZ compared to varicella (Brisson *et al.*, 2001). However, because HZ occurs mainly in the elderly compared to varicella, the overall burden on in-patient hospitalization for HZ is reported to be considerably higher (Brisson *et al.*, 2001; Brisson and Edmunds, 2003; MacIntyre, 2003). In a US study of HZ hospitalizations, the authors did not differentiate between primary vs coincidental cause of HZ hospitalization or death (Lin and Hadler, 2000). Coincidental cases of HZ may be quite common especially among elderly hospitalized adults with long hospital stays, some of whom will die from other causes (MacIntyre *et al.*, 2003). This may contribute to overestimation of the health burden due to HZ.

Several studies reported lower HZ rates among persons exposed to varicella cases (Solomon *et al.*, 1998; Terada *et al.*, 1995; Thomas *et al.*, 2002), compared to those without any exposure. Thomas showed that contacts with ≥5 varicella cases were associated with a strong protective effect against HZ after controlling for occupational and social (OR = 0.29, 95% CI 0.10–0.8) compared to those with no contacts. The two other smaller studies with methodological limitations reported that pediatricians had lower HZ incidence than the general population (Terada *et al.*, 1995), and dermatologists or psychiatrists (Solomon *et al.*, 1998). Terada based his findings on small numbers of cases; while Solomon's findings were based on low response rates (31% paediatricians) and found no differences between dermatologists and psychiatrists, groups considered to have different rates of exposure to varicella cases. Finally, in all three studies, protection was observed for groups with more exposure to varicella than is generally experienced by the general population.

Other studies have used exposure to children as a proxy for exposure to varicella. Two observational studies in England reported that household or occupational exposure to children was associated with a lower HZ incidence (Brisson *et al.*, 2002; Thomas and Hall, 2004). Brisson reported that people living with children had a significantly lower rate of developing HZ (RR, 0.75 95% CI 0.63–0.89). In a subsequent analysis of the same data, Thomas reported that individuals who reported working with young children (primary school teachers, nursery nurses, playgroup leaders and other child care providers) were significantly less likely to develop HZ after adjusting for age, sex, ethnicity and a child living in the household (RR, 0.70, 95% CI 0.58–0.85). However, it is not clear how other factors that could have contributed to such findings were adjusted for in the analysis including the fact that people with other medical health problems, who are at a higher risk for HZ, are less likely to live with children. Furthermore, Thomas and Hall (2004) in their review of risk factors for HZ report that women in general have higher incidence rates of HZ compared to males; they comment that these findings conflict with the suggested reduced HZ risk associated with exposure to varicella cases or children.

The protection against HZ due to varicella exposure is plausible and is supported by immunologic studies. One reported that 71% of adults who had household exposure to varicella experienced a boost in cellular immune responses (Arvin *et al.*, 1983), and the other found that vaccinated leukemic children with household exposure to varicella were less likely to develop HZ than vaccinated leukemic children without such an exposure (Gershon *et al.*, 1996; Hardy *et al.*, 1991). Yet, other issues still remain unknown, including factors that contribute to immune boosting, the duration of protection from such exposures, and other factors that influence VZV reactivation. Herpes zoster affects mainly the elderly, and if there is a protection from exposure to varicella, the duration of this protection is not known. However, modeling, based on the assumption that the boosting effect lasts for 20 years, suggests that an increase in HZ incidence will occur as early as five (Garnett and Ferguson, 1996) to seven (Brisson *et al.*, 2002) years following the implementation of a mass vaccination program.

Herpes zoster in vaccinated children

Current data suggest that vaccinated children experience a lower HZ incidence rate than those who have had wild varicella. Herpes zoster incidence among healthy children who received varicella vaccine is reported to be rare; the incidence of HZ among these children was reported to be approximately 13 cases per 100 000 person–years (Gershon *et al.*, 2004*)* however, longer follow-up is needed. Furthermore, studies in leukemic children have found a much lower incidence of HZ among vaccinated (2%) than among age matched children with a history of varicella (15%) (Hardy *et al.*,1991). Therefore, it is expected that HZ incidence will decline over the long term, as vaccinated cohorts replace those in the community with naturally acquired varicella.

Vaccination for prevention of herpes zoster

As populations age and the survival of people with chronic and immunocompromised conditions improves, the incidence of HZ and PHN is expected to increase. Several studies have suggested that declines in VZV-specific CMI increase the risk and severity of HZ (Arvin 1996; Miller, 1980; Oxman, 1995). Early studies on administration of a higher titer varicella vaccine to older adults 55+ have shown that vaccinated persons experience an increased VZV-specific CMI responses to levels typical of those observed in younger persons, in whom the incidence and severity of HZ are lower (Levin *et al.*, 1998; Levin, 2001). A recent study reported on the boosting in VZV-specific cell-mediated immunity from a booster dose of VZV vaccine administered ≥5 years after the first dose (Levin *et al.*, 2003). In 2005, the results of a large placebo-controlled clinical trial to test whether a shingles vaccine in persons 60 years and older would prevent or reduce the risk or severity of HZ and its complications became available. The vaccine was 51.3% efficacious in reducing herpes zoster incidence, 66.6% efficacious in reducing the incidence of postherpetix neuralgia (largely through preventing herpes zoster) and 61.1% efficacious in reducing the burden of illness due to herpes zoster (Oxman *et al.*, 2005). This vaccine, now licensed in the USA with expected licensure in other countries, has the potential to reduce the incidence of HZ or reduce/attenuate the severity of PHN, and complications of HZ. (Johnson *et al.*, 2002; Schmader, 2001; Gilden, 2005). In the U.S. Zoster vaccine was recommended for use in all adults ≥60 years without a contraindication in October 2006 (CDC, 2006).

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Part III

Pathogenesis, clinical disease, host response, and epidemiology: betaherpesviruses

Edited by Ann Arvin and Richard Whitley

41

Virus entry into host, establishment of infection, spread in host, mechanisms of tissue damage

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Introduction

Cytomegaloviruses (CMV) were initially identified by distinct histopathological findings that were observed in tissue from a variety of infected mammals, including humans. Perhaps the most well-recognized finding were inclusion bearing cells in the salivary glands of infected animals (Jesionek and Kiolemenoglou, 1904; Ribbert, 1904; Goodpasture and Talbot, 1921; Cole and Kuttner, 1926). Similar histopathologic findings of intracellular inclusions were noted in tissues from infants dying as a result of severe congenital (present at birth) cytomegalovirus infection leading to the designation of this clinical syndrome as cytomegalic inclusion disease (Farber and Wolbach, 1932). Studies by several groups of investigators provided compelling evidence from natural history studies that HCMV was a relatively frequent cause of disease in infants infected in utero and, that this viral infection could result in neurologic impairment in infected infants (Hanshaw, 1971; Stagno *et al.*, 1977; Pass *et al.*, 1980; Williamson *et al.*, 1982; Bale, 1984; Fowler *et al.*, 1992). Importantly, these early studies demonstrated that even infants with subclinical or silent infections could develop neurological sequelae (Stagno *et al.*, 1982, 1983; Williamson *et al.*, 1992). In the late 1960s, HCMV was recognized as a significant cause of disease in allograft recipients and in the case of hematopoietic allograft recipients, HCMV infection became recognized as one of the most frequent causes of death in the post-transplant period (Rifkind, 1965; Myers *et al.*, 1975; Ho, 1977; Rubin *et al.*, 1979; Winston *et al.*, 1979; Rubin *et al.*, 1981; Rubin and Colvin, 1986; Rubin, 1990). Significant morbidity and mortality rates were reported in allograft recipients infected with HCMV until efficacious antiviral chemotherapy was developed for this agent (Emanuel *et al.*, 1988; Schmidt *et al.*, 1991; Goodrich *et al.*, 1993; Winston *et al.*, 1993). Similarly, HCMV rapidly emerged as a major opportunistic pathogen in patients with HIV infection, particularly those in the late stages of the retroviral infection (Klatt and Shibata, 1988; Gallant *et al.*, 1992; Bowen *et al.*, 1995; Selik *et al.*, 1996; Spector *et al.*, 1999). The spectrum of diseases associated with infection with HCMV has been well described in immunocompromised patients with acute infectious syndromes in which virus replication can be correlated with end-organ disease. Because immunocompromised patients often present with multiorgan dysfunction secondary to chronic underlying disease and in some cases the pharmacologic agents utilized to treat allograft rejection, it has been difficult to completely define the spectrum of clinical disease associated with HCMV infection. In contrast to findings in allograft recipients, the disease manifestations of congenitally (present at birth) infected infants can be related directly to HCMV infection. Yet even in this group of patients the contribution of organogenesis particularly that of the developing central nervous system, to the pathogenesis of disease in HCMV infected infants is not completely understood. As a result, it is sometimes difficult to directly translate the clinical features of congenital HCMV infections and end-organ disease to clinical syndromes observed in other patient populations such as allograft recipients. Lastly, it should be stressed that, in nearly all cases, symptomatic disease following acute HCMV infection is limited to patients with deficits in their immune system and clinical evidence of acute HCMV infection is rarely seen in normal hosts.

In contrast to the obvious role of HCMV in acute infectious syndromes in immunocompromised hosts, the role of this agent in chronic disease syndromes remains controversial even after decades of study. Early studies suggested a role of HCMV in several chronic diseases that are more prevalent in middle-aged and older individuals such as coronary atherosclerosis (Petrie *et al.*, 1988). In addition, epidemiological studies have suggested a link

between infection with HCMV and human cancers (Rapp and Robbins, 1984; Shen *et al.*, 1993; Hsieh *et al.*, 1999). More recent studies have provided convincing evidence for a role of HCMV in the accelerated vascular disease that is observed in solid organ allograft recipients, a disease process that may represent the extreme of the spectrum of HCMV-induced vascular disease (Grattan *et al.*, 1989; Melnick *et al.*, 1993; Epstein *et al.*, 1996; Zhu *et al.*, 1999). Recent studies have also demonstrated HCMV nucleic acids and viral proteins in human cancers, providing provocative evidence for a potential role of this virus in malignant behavior of at least some human tumors (Geder *et al.*, 1977; Shen *et al.*, 1993; Cobbs *et al.*, 2002; Harkins *et al.*, 2002). The life-long persistence of the virus in the infected host, the ubiquitous nature of the infection in the population, and the recently recognized disruption of normal host cellular functions suggests that careful investigations into the role of this virus in chronic diseases should remain a major focus of research in diseases associated with HCMV infection.

Acquisition of HCMV: Sources of virus and transmission within populations

Human CMV infection, as defined by serological evidence of previous infection, is ubiquitous in most populations with HCMV seroprevalence ranging from 20%–100% in different regions of the world (Alford *et al.*, 1981; Krech and Tobin, 1981; Gold and Nankervis, 1982). In general, individuals from resource constrained countries have an increased HCMV seroprevalence and studies from sub-Saharan Africa, South America, and India have suggested that by early adulthood nearly 100% of individuals have been infected with HCMV (Alford *et al.*, 1981). In contrast, in well-developed countries in Northern Europe and North America, the seroprevalence can range from 20%–100% in adults. Seroprevalence studies in the United States, Great Britain, Italy, and Scandinavia have provided clues as to routes of virus acquisition and sources of exposure in the community (Weller, 1971). Similarly, careful epidemiological studies aided by genetic analysis of virus isolates have helped define sources of virus infection in young children attending child-care centers, hospitalized patients and hospital staff.

Sources of HCMV in the community

Infectious virus has been recovered from saliva, tears, breast milk, semen, blood products, urine, and cervical secretions. With the exception of blood products, persistent virus infection of epithelial cells in secretory glands remains a constant theme for HCMV infection. Although the phenomena of virus persistence in the salivary glands of small animal models of the HCMV infection have been intensively studied, mechanisms leading persistence of the virus in epithelial cells of apacrine glandular tissue remain incompletely described. Similarly, the question of latency with abortive virus replication vs. a chronic persistent productive infection in these tissues has not been resolved. Studies in animal models and in vitro cell culture system indicate that latent infection can occur, yet these findings have been validated in only limited types of human cells (Jordan and Mar, 1982; Jordan *et al.*, 1982; Kondo *et al.*, 1996; Sinclair and Sissons, 1996; Kurz *et al.*, 1997; Soderberg-Naucler *et al.*, 1997; Hahn *et al.*, 1998; Kurz *et al.*, 1999; Goodrum *et al.*, 2002). Regardless of whether the virus is maintained as a latent infection with periodic reactivation or as a chronic persistent infection yielding low titers of infectivity, the virus readily spreads within a population. Characteristics of HCMV infection such as prolonged virus shedding that may last for over 6 months in some individuals with acute HCMV infection favor spread within susceptible populations. In young infants, HCMV infection acquired in the perinatal period or even during infancy can result in the virus excretion lasting several years (Stagno *et al.*, 1975a,b). Thus, in contrast to periods of communicability for respiratory viruses that are often measured in weeks, acutely infected hosts can remain contagious for months to years after HCMV infection. Although the mechanisms underlying its persistence are far from understood, during its evolution with its human host the virus has developed multiple strategies for persistence in the host and in populations. Persistence in the population has been accomplished without the necessity for a secondary animal reservoir or non-human vectors for transmission.

Although infection with HCMV has been shown to be endemic in all populations studied in the world, transmissibility of the virus has been suggested to be limited based on observations that close contact is required for infection within a population. Even though the efficiency of transmission may be limited either secondary to the liability of the virus or low levels of infectious virus excretion, its persistence favors repeated exposures of susceptible hosts to infectious virus. This is perhaps best illustrated by the finding that in almost all reported studies, acquisition of HCMV serological reactivity increases with age (Alford *et al.*, 1981). In developed countries, primarily Northern Europe and North America, numerous serological studies have been carried out and together with more limited findings in populations from the developing world, have provided some understanding of the sources of HCMV in the community and modes of virus infection. In the developed

Table 41.1. Sources of community-acquired HCMV infections

Source	Infectious fluid	Mode of transmission
Pregnant women	Blood; genital secretions	Blood-borne transmission to Fetus; intrapartum ingestion of infected genital secretions
Lactating women	Breast milk	Ingestion of cell free virus by breast feeding infants
Young children	Saliva; urine	Ingestion
Adolescents/adults	Saliva; genital secretions	Ingestion; sexual contact

Table 41.2. Relationship between age and acquisition of HCMV. Estimated age for >50% seropositivity of population based on published seroprevalence and age

Location	Age at which 50% seropositive
Chile	6 mo–1 yr
Ivory Coast	< 1 yr
Birmingham, Al (low SES)	4–9 yrs
Rochester, NY (high SES)	>28–35 yrs
Tanzania	< 1 yr
India	1 yr
London	> 28–35

From Alford *et al.*, 1981.

world, the variation in the prevalence of HCMV infection has been related to age, child-care practices, sexual activity, and socioeconomic status. These epidemiological associations are consistent with the finding of infectious virus in saliva, tears, breast milk, semen, urine, and cervical secretions (Table 41.1).

Serological evidence of HCMV infection has been used as a measure of exposure to HCMV in different population. Because no specific genetic linkage has been associated with susceptibility or resistance to HCMV infection, it follows that increasing rates of serologic reactivity reflect the relative risk of HCMV exposure of individuals in that population. When HCMV sero-prevalence is compared to age in different populations, it is apparent that near universal seroreactivity in young adults from less developed regions of the world are also associated with early acquisition of HCMV (Table 41.2).

In some parts of the world such as sub-Saharan Africa and southern Asia, nearly 100% of young children are seropositive for HCMV suggesting that exposure to HCMV is universal in these populations (Alford *et al.*, 1981). In contrast, early studies in North America and Northern Europe suggested that between 10–50% of young children were seropositive for HCMV (Alford *et al.*, 1981). Increased rates of serologic evidence of HCMV infection have been noted in children from lower socioeconomic groups in the USA, although these rates do not approach those observed in similarly aged children in the developing world. In addition, increased rates of seropositivity were noted in children from populations in which breast feeding was common and in populations in which group child care was commonly utilized (Weller, 1971). There is no single explanation that accounts for the differences in the age of HCMV acquisition that has been observed in children from Northern Europe and North America and young children from the southern hemisphere. The most frequently offered explanation is that increased rates of infection in Africa and Asia are secondary to near universal breast feeding and possibly to overcrowding in homes with young children (Stagno, 1995a,b). These explanations are consistent at least with observations from child care centers in the USA which have demonstrated an increased rate of virus transmission between children attending child care centers (Pass *et al.*, 1984; CDC, 1985; Hutto *et al.*, 1985; Murph *et al.*, 1986; Adler, 1988; Dobbins *et al.*, 1993). The other component of this explanation is the observed efficient spread of HCMV through breast milk to nursing infants (Stagno *et al.*, 1980; Dworsky *et al.*, 1983a,b; Ahlfors and Ivarsson, 1985; Minamishima *et al.*, 1994; Vochem *et al.*, 1998). Breast milk has been shown to contain significant amounts of infectious virus, much of which is cell free and readily transmitted (Asanuma *et al.*, 1996; Vochem *et al.*, 1998). Studies have documented that over 60% of breast fed infants born to a HCMV infected mother will become infected with HCMV (Stagno *et al.*, 1980; Dworsky *et al.*, 1983). This rate likely underestimates the efficiency of breast milk transmission, particularly in populations in which infants are exclusively breast fed. Because young infants infected following ingestion of infected breast milk excrete significant amounts of virus for extended periods of time, infected infants readily transmit virus to household and community contacts. The rate of breast feeding in countries from the developing world far exceed those in many populations in North America and Northern Europe, particularly when compared to the decreased rates of breast feeding in women from lower socioeconomic groups in the USA. Thus, it is likely that early childhood acquisition of HCMV in populations with high rates of HCMV infection in women of childbearing age can be explained by exposure to virus containing breast milk coupled with crowded living conditions that favor spread

of HCMV among young children and other members of the household.

Young children as a source of HCMV

Young children have also been documented to be an important reservoir of infectious virus in the community. Transmission of HCMV between young children appears to be very efficient, presumably because of frequent physical contact, repeated hand-to-mouth contact, and the limited personal hygiene practices of infants and toddlers (Hutto *et al.*, 1986). The initial speculation by Weller that high rates of infection in Swedish children as compared to infants in New York was secondary to the increased use of group child-care centers in Sweden was subsequently confirmed by a number of studies (Weller, 1971). Studies carried out in child-care facilities in the United States have consistently documented increased rates of HCMV infection in young children, particularly those less than 18 months of age (Table 41.2) (Pass *et al.*, 1984; CDC, 1985; Murph *et al.*, 1986; Pass *et al.*, 1987; Adler, 1991a,b). In a study of a group care center that enrolled infants of middle and upper-middle class parents, the seroprevalence in the infants increased from about 10% in the first year of life to over 80% in the second year of life (Pass *et al.*, 1982a,b). Similar results were reported by Adler *et al.* who also noted that over 50% of initially seronegative infants acquired a day-care related strain of HCMV over a 26-month period as determined by restriction fragment length polymorphism (RFLP) of viral DNA (Adler, 1985). In addition, this study as well as other studies has documented HCMV shedding in 20%–40% young children attending day-care centers (Pass *et al.*, 1987). The ease with which HCMV can be transmitted between young children together with the prolonged duration of virus excretion in infants with congenital and perinatal infections attending child-care centers provides an ideal setting for spread of this virus to young children in group care facilities. Furthermore, infants infected following exposure in child-care centers also excrete significant amounts of virus for extended periods of time, increasing the reservoir of transmissible virus in a group-care setting. Thus, group child-care settings could mimic the exposure of young infants in developing countries in which child-care is often shared among members of the community. The routes of HCMV transmission between children has not been defined but is thought to be primarily through hand-to-mouth contact.

Young children not only readily transmit HCMV to other children but also serve as an important source of HCMV infection of adults (Taber *et al.*, 1985; Pass *et al.*, 1986; Adler, 1988, 1989, 1991; Murph *et al.*, 1991). Early studies utilizing RFLP clearly demonstrated that HCMV could be transmitted from young children to adults (Spector and Spector, 1982; Dworsky *et al.*, 1984). Similarly, several epidemiologic studies related exposure to young children with acquisition of HCMV (Yeager, 1983). These studies have documented increased annualized seroconversion rates in women exposed to young children as compared to women in homes without young children. Later studies included the parents of children attending child-care centers and at least two groups of investigators documented infections in 33%–45% for women whose children were shedding HCMV (Pass *et al.*, 1986; Adler, 1988). Interestingly, the increased rates of virus infection were not limited to the parents of children attending child-care centers but were also noted in workers in child-care centers (Adler, 1989; Pass *et al.*, 1990; Dobbins *et al.*, 1993). An approximate five fold increase in the rate of HCMV infection as measured by seroconversion was noted in one group of women employees at child care centers (Adler, 1989). Analysis by RFLP of DNA from viruses isolated from children in the childcare centers and infected workers revealed similar patterns, suggesting a common source of infection (Adler, 1989). Although it is clear that child-care workers are at risk for acquisition of HCMV secondary to their exposure to young children, an increased risk of HCMV infection has not been demonstrated in hospital workers, including nurses caring for infants with perinatal HCMV infections (Yeager, 1975; Dworsky *et al.*, 1983a,b; Adler *et al.*, 1986; Balfour and Balfour, 1986). These findings argued that with adequate attention to routine hygienic practices, the risk for acquisition of HCMV is no greater for individuals caring for children than that of the general population. Finally, in several prospective natural history studies, maternal HCMV infection and the delivery of a congenitally infected baby have been linked to exposure to young children (Pass *et al.*, 1987; Fowler *et al.*, 1997). Thus, HCMV-infected children are efficient vectors for transmission of the virus to other children and their caretakers because they serve as a reservoir of virus by shedding virus for a prolonged period and because their behavior facilitates virus spread to susceptible contacts.

Acquisition of HCMV by sexual contact

As described above, HCMV infection occurs at an early age in populations in the developing world and virus acquisition through sexual contact is likely of limited importance in primary infection of HCMV in these populations. In contrast, in some populations in North America and northern Europe, a second rapid increase in HCMV seroprevalence has been observed during adolescence, suggesting that virus is acquired through sexual contact. A large number of epidemiological studies have supported the classification

of HCMV as a sexually transmitted infection (STI). The associations between sexual activity and HCMV infection are listed in Table 41.3.

Studies of women attending sexually transmitted disease clinics have noted an increase in both HCMV seroprevalence and definitive evidence of HCMV transmission between sexual partners (Jordan *et al.*, 1973; Chandler *et al.*, 1985, 1987; Handsfield *et al.*, 1985; Collier *et al.*, 1990; Coonrod *et al.*, 1998). In addition, it appeared that previously infected individuals could be re-infected by a second strain of virus (see following section) (Handsfield *et al.*, 1985). Other associations between sexual activity and HCMV infection have included: (1) number of sexual partners, (2) number of lifetime sexual partners, and (3) co-infection with other sexually transmitted infections such as trichomonas, gonorrhea, and bacterial vaginosis (Chandler *et al.*, 1985, 1987; Handsfield *et al.*, 1985; Collier *et al.*, 1990; Fowler and Pass, 1991; Sohn *et al.*, 1991; Coonrod *et al.*, 1998). Similarly, in a sexually active male homosexual population it has been reported that over 90% of these men were infected with HCMV (Drew *et al.*, 1981; Drew and Mintz, 1984).

The association between HCMV infection and sexual activity is consistent with the shedding of this virus in the genitourinary tract. Both cervical secretions and vaginal fluid are frequent sites of virus recovery in populations of younger women (Reynolds *et al.*, 1973; Stagno *et al.*, 1975a,b; Waner *et al.*, 1977; Collier *et al.*, 1995; Coonrod *et al.*, 1998). Semen has been reported to be a rich source of virus and quantitation of viral DNA in semen has been used to monitor the in vivo response to antiviral drugs in clinical studies (Lang and Kummer, 1975; Drew and Mintz, 1984 ; Rinaldo *et al.*, 1992; Yang *et al.*, 1995; Liesnard *et al.*, 1998; Diamond *et al.*, 2000). The frequency with which HCMV can be isolated from the female genital tract varies between populations and interestingly, with the age of the woman (Collier *et al.*, 1995; Coonrod *et al.*, 1998). In some studies virus could be recovered from the genital tract of over 50% of women attending a STD clinic, whereas the rate of recovery from HCMV infected women in the post partum period were significantly lower (Pass *et al.*, 1982a,b). Thus, it is unclear if the increased rates of recovery of HCMV from women attending an STD clinic reflect increased shedding from a previous chronic infection or repeated re-infections. A second interesting observation is that age appears to influence the frequency of HCMV shedding in the genital tract of women such that young women infected with HCMV are more likely to shed virus from the genital tract as compared to older women (Fowler *et al.*, 1993; Collier *et al.*, 1995; Coonrod *et al.*, 1998). However, the relationship between risk of re-exposure to HCMV through sexual contact in these two populations and rates of genital shedding of virus remains inadequately defined. Alternatively, it has been argued that primary HCMV infection leads to more prolonged shedding of virus and the more frequent recovery of virus from the genital tract of younger women could reflect the natural history of a resolving primary infection. These two possible explanations have not been reconciled.

Table 41.3. Representative studies that have associated HCMV infection with sexually transmitted infections

Population	Evidence of sexual transmission
Sexually active male homosexuals	Increased seroprevalence (> 90% +) as compared to general population (Drew *et al.*, 1981).
Women attending STD clinic	Increased seroprevalence; increased rate of cervical shedding; increased rates of seroconversion (Chandler *et al.*, 1985; Handsfield *et al.*, 1985; Collier *et al.*, 1990; Coonrod *et al.*, 1998).
Young women	HCMV infection associated with STIs including bacterial vaginosis, trichomonas and gonorrhea (Chandler *et al.*, 1985; Fowler and Pass, 1991; Sohn *et al.*, 1991)
Young women	Increased rates of HCMV seroconversion correlated with increased number of sexual partners (Collier *et al.*, 1990; Fowler and Pass, 1991)

Sources of HCMV in hospitalized patients and health-care workers

Some of the earliest reports of the clinical syndromes associated with HCMV infection described patients infected with HCMV by nosocomial routes. Several routes of nosocomial transmission have been described but the three most frequently routes of transmission include: (1) allograft transplantation, (2) blood products, and (3) breast milk. Transfusion associated HCMV infection was recognized in cardiac surgery patients who in the past often required significant quantities of blood as a result of cardiac bypass procedures. A syndrome of fever, atypical lymphocytosis, elevated liver transaminases, and splenomegaly was associated with these procedures and the phrase post-perfusion syndrome or post-perfusion mononucleosis was coined (Holsward *et al.*, 1963; Reyman, 1966; Paloheimo *et al.*, 1968). Subsequent studies revealed that HCMV infection could be related to up 50% of these cases and it was argued that HCMV was transmitted by blood products utilized in these procedures (Prince *et al.*, 1971). From a series of studies in seronegative patients receiving blood products it was estimated that risk of acquiring HCMV infection was about 2.5% per unit of blood transfusion (Stevens *et al.*, 1970; Armstrong *et al.*, 1976; Bowden, 1995). Because the total amount of blood products also correlated with the risk of acquiring HCMV, patient populations

requiring large amounts of blood and blood products secondary to their underlying disease or complications of therapy were at increased risk for HCMV infection. In addition, the type of blood product transfused also influenced risk for transmission of HCMV. Studies in allograft recipients revealed that blood products that contain white blood cells or platelets were more likely to transmit HCMV to the recipient. As a result, several modifications were introduced to limit white blood cell contamination of blood, including the use of filters that can remove white blood cells from red blood cell transfusions and the use of frozen red blood cell preparations (Brady *et al.*, 1984; Bowden *et al.*, 1991; Ljungman *et al.*, 2002a,b). Both approaches have been shown to reduce the incidence of transfusion acquired HCMV infection. The second approach involved screening blood donors and selecting those without serological evidence of past HCMV infection. This approach has proven successful in reducing the incidence of HCMV infection in transfusion recipients, particularly in bone marrow allograft recipients (Bowden *et al.*, 1991; Bowden, 1995). However, two limitations have become apparent. The first is that in some donor populations over 50% of patients have serological evidence of HCMV infection, thus restricting the donor population and limiting the availability of blood products. The second more recent observation is that perhaps up to 15% of donors without serological evidence of HCMV infection can be shown to have evidence of previous HCMV infection when analyzed by more sensitive techniques such as PCR (Roback *et al.*, 2001; Drew *et al.*, 2003). The interpretation of this interesting finding is not straightforward as it is unclear what proportion of blood products from these serologically negative, PCR positive donors transmitted HCMV to recipients.

HCMV infections resulting from transfusions of blood products have also been shown to cause significant disease in newborn infants, particularly premature infants and infants born to women without serological immunity to HCMV (Yeager *et al.*, 1981; Adler, 1983; Adler *et al.*, 1983). Because extremely premature infants, regardless of their mothers' HCMV serologic status, are delivered prior to significant transplacental transfer of maternal antibodies, they often lack passively acquired antibody immunity to HCMV. Thus, these infants are at risk for severe HCMV infections. Perhaps one of the more interesting findings from these studies of nosocomial infections in premature infants was that passively acquired anti-HCMV antibodies provided some protective immunity. In a prospective study, infected infants with antiviral antibodies exhibited a milder disease than infected infants without passively acquired anti-HCMV antibodies and appeared to be protected from severe HCMV infections and in some cases, death (Yeager *et al.*, 1981). This observation also provided one of the most compelling arguments for a role of antiviral antibodies in host protective responses to HCMV that limit disease.

Breast milk transmission of HCMV is well described (see above) and infection of extremely premature infants as a result of ingestion of infected breast milk can be associated with severe disease (Vochem *et al.*, 1998). The finding that breast milk transmitted HCMV could result in significant disease in young infants defined an additional risk in the use of banked breast milk as nutritional support for premature infants. The lack of antiviral antibodies in premature infants was associated with symptomatic infection following breast milk acquisition of HCMV similar to infections associated with transfusions. Even though breast milk from HCMV seropositive women contains virus neutralizing antibodies of both the IgG and IgA isotypes, virus can be readily transmitted to infants. This finding suggests that antiviral antibodies cannot prevent virus transmission even if present in the inoculum.

Transmission of HCMV by transplantation of an allograft from donor previously infected with HCMV represents a major clinical problem in allograft transplantation. Human cytomegalovirus is the most common cause of infection in the post transplant period and the transplanted allograft represents the most important source of HCMV infection in allograft recipients (Peterson *et al.*, 1980; Rubin 1990; Rubin and Colvin, 1986, Griffiths *et al.*, 2000; Ljungman, 2002; Ljungman *et al.*, 2002a,b). It is estimated up to 90% of allograft recipients who have received an allograft from a HCMV infected donor will become infected with HCMV; however, it is likely that all recipients of allografts from HCMV infected donors will become infected with HCMV present in the donor allograft, regardless of their serological status prior to transplantation. Although prior experience with HCMV either as the result of natural infection or live virus vaccines has been shown to provide some protection from severe infection and end-organ disease, pre-existing immunity does not prevent infection (Plotkin *et al.*, 1991). Viral nucleic acids have been detected in the kidneys from HCMV infected donors indicating that the transplanted organ can serve as a source of virus in transplanted allografts (Gnann *et al.*, 1988). In addition, bone marrow allografts from HCMV seropositive donors readily transmit HCMV to recipients, a finding consistent with the demonstration that the cells of macrophage/monocyte lineage can harbor latent HCMV (Soderberg-Naucler *et al.*, 1997). Reactivation of latent HCMV from macrophages derived from HCMV seropositive donors has been demonstrated in-vitro, providing an explanation for the transmission of HCMV from both hematopoietic allografts as well as solid organs that almost certainly contain contaminating macrophages (Soderberg-Naucler *et al.*, 1997). Together with transfusion acquired

HCMV infection, infection in allograft recipients from the virus in allograft represent the most common mode of nosocomial transmission of HCMV.

Transmission of HCMV from patients shedding HCMV to health-care workers is exceedingly rare. Shedding of significant titers of infectious virus is usually limited to hospitalized infants and young children and studies have shown that nurses exposed to these infected infans are no more likely to become infected with HCMV than control populations in the community (Dworsky *et al.*, 1983; Adler *et al.*, 1986; Demmler *et al.*, 1987). In the case of adults, HCMV shedding in hospitalized patients is usually only observed in severely immunocompromised patients such as allograft recipients and patients with AIDS. With the implementation of universal precautions in the care of hospitalized patients, the risk of nosocomial transmission of HCMV to a healthcare worker is almost certainly lower than the risk of community acquisition of this virus.

Reinfection: acquisition of HCMV by a previously infected host

Superinfection or reinfection of previously infected immunocompromised allograft recipients has been well described (Chou, 1986, 1987; Grundy *et al.*, 1988). In addition, numerous studies in patients infected with HIV demonstrated multiple infections with HCMV and in some patients, several genetically distinct strains of HCMV could be detected simultaneously (Drew *et al.*, 1984; Spector *et al.*, 1984; Chern *et al.*, 1998). These studies provided evidence that pre-existing immunity to HCMV, both antiviral antibodies and HCMV-specific cellular immune responses, failed to prevent infection with an unrelated strain of HCMV. Anecdotal reports have suggested that normal seropositive individuals could be superinfected with new strains of HCMV; however, it was argued that reinfections were only common in immunocompromised hosts. A study of vaccine immunity following inoculation with a candidate replicating virus vaccine demonstrated that individuals infected with the vaccine strain of HCMV could be reinfected with a challenge strain of HCMV (Plotkin *et al.*, 1989). A more recent study also demonstrated that normal women infected with a vaccine strain of HCMV could be reinfected following natural exposure to young children shedding HCMV (Adler *et al.*, 1995). These studies provided definitive evidence that prior infection in a normal host could not prevent a second infection (Table 41.4). In this latter report, the authors speculated that reinfection resulted from a failure of the vaccine to induce immunity that was similar to that following natural infection with a wild type strain of HCMV (Adler *et al.*, 1995).

Table 41.4. Infection in normal hosts with preexisting immunity to HCMV. Studies demonstrating the acquisition of a second strain of HCMV in a previously infected host

Population	Evidence of reinfection
Vaccine recipients	Clinical symptoms with virus challenge; virus excretion with virus challenge (Plotkin *et al.*, 1989).
Vaccine recipients	serologic boost in previously infected host following community acquired infection; recovery of non-vaccine strain of virus in recipients of live virus vaccine (Adler *et al.*, 1995)
Infants in day care	recovery of genetically distinct viruses over time from infants in day care center (Bale *et al.*, 1996)
Women with congenitally infected infants	serological evidence of acquisition of new gentoype in women with prior HCMV infection; delivery of infected infant following reinfection (Boppana *et al.*, 2001a,b)
experimental animals	serologic and virologic evidence of reinfection in both wild and captive mouse populations; reinfection in captive non-human primates (Moro *et al.*, 1999)

More recent findings in adults as well studies in young children have demonstrated that individuals with prior HCMV immunity can be readily reinfected with a second (or possibly third, fourth, etc.) strain of HCMV as a result of natural exposure. The spread of HCMV in young children attending a child care facility clearly demonstrated that previously infected young children could be reinfected with a second strain of HCMV (Bale *et al.*, 1996). More recently, reinfection of normal women by a genetically unrelated strain of HCMV was studied utilizing an antigenic polymorphism in glycoprotein H that allowed the detection of reinfection by serologic reactivity (Boppana *et al.*, 2001a,b). In this study, reinfection of women previously infected with HCMV with a new viral strain that encoded a unique serologic determinant was shown to occur in nearly 30% of women enrolled in the study (Boppana *et al.*, 2001a,b). Interestingly, reinfection in this population was associated with intrauterine transmission of HCMV during subsequent pregnancies and damaging congenital infection was observed in three infants infected in utero as a result of maternal reinfection (Boppana *et al.*, 2001a,b). Subsequent studies in this same population have indicated that approximately 12% of women are reinfected with a second virus strain each year. It is important to note that the serologic methods utilized in this study likely underestimates the frequency of reinfection because only two antigenic variants could be detected (Boppana *et al.*, 2001a,b). Because of this limitation, it is highly likely that women in this population are frequently reinfected with genetically

different strains of HCMV. In view of the reported case of recombination between strains of HCMV, some individuals may harbor HCMV of significant genetic diversity, perhaps approaching a so-called swarm of viruses.

The importance of reinfection as a source of HCMV in a population is unclear but the ease with which HCMV can spread within populations and the apparent lack of protective immunity suggests that reinfection with new strains of HCMV are common in all populations and dependent only on the risk of exposure to infectious virus. In experimental animal model systems reinfection has been documented, including in the rhesus macaques (personal communication, J. Nelson, Oregon Health Sciences University, Portland, Or.) and mice. The spread of murine CMV in wild mice has been documented and multiple genetically unique strains of viruses have been found in a single mouse, indicating that multiple infections also occur in normal mice (Moro *et al.*, 1999). Thus, when the spread of HCMV in populations is defined it is critical to include not only seronegative individuals, a group previously viewed to be the sentinel for HCMV spread in a population, but also seropositive individuals as both populations can be readily infected with HCMV. The design and strategy for vaccine control of HCMV must keep these possibilities in mind.

Entry and spread within susceptible hosts

Infection following community exposure

Infection with HCMV following community exposure presumably occurs as a result of exposure of mucosal surfaces of the upper respiratory tract or the genital tract to infectious HCMV. Although the cellular target of HCMV infection remains incompletely defined, the widespread expression of putative cell surface receptors including, proteoglycans, integrins, and epidermal growth factor receptor, suggest that the tropism of virus is not limited to specific cell types on the mucosal surface. Presumably virus attaches and enters susceptible epithelial cells and undergoes lytic replication. Following the release of progeny virions, adjacent cells including non-epithelial cells of the underlying submucosa are infected. This earliest phase of cytomegalovirus infection has not been well studied in humans or experimental animal models and the pathway of infection and local amplification that is postulated is based on modes of virus exposure that lead to infection. The steps following local amplification leading to disseminated spread of virus to the liver and spleen and eventually to sites of persistence are essentially unknown in humans. Studies in experimental animals including mice and primates have demonstrated that infection of the oral mucosa leads to dissemination and widespread infection of organs such as the liver and spleen (Kern, 1999; Lockridge *et al.*, 1999). In a primate model of HCMV infection, Lockridge *et al.* observed that both intravenous and oral inoculation of rhesus macaques with rhesus CMV led to dissemination and widespread viral infection in a variety of organs (Lockridge *et al.*, 1999). Perhaps the most surprising observation made in this study was that oral inoculation lead to virus dissemination and infection of liver and spleen as efficiently as intravenous inoculation of equivalent amounts of infectious virus (Lockridge *et al.*, 1999). However, the kinetics of virus spread for the intravenously inoculated animals was accelerated as compared to the orally infected animals (Lockridge *et al.*, 1999). Because peak of viremia was not detected until approximately 1 week later in the animal inoculated by mucosal exposure as compared to those given virus intravenously, it could be argued that following mucosal exposure, the virus undergoes a local amplification and then spread to secondary sites of infection such as the liver and spleen and regional lymph nodes (Lockridge *et al.*, 1999). Studies in other experimental models such as mice and guinea pigs are consistent with this mode of spread in the host (Bernstein and Bourne, 1999; Kern, 1999). Thus, it appears that HCMV infection could follow a similar pathway of infection and spread as has been described for varicella-zoster virus (Grose, 1994). After mucosal infection with HCMV there is local amplification, an initial viremia that leads to infection of visceral structures such as the liver and spleen. Infection of these organs is then followed by a secondary viremia that leads to a more generalized infection. From studies in experimental animals and transfusion acquired HCMV infections, it is clear that the magnitude of the inoculum, the kinetics of virus replication at the primary site, and the host derived innate responses can influence the kinetics, duration, and magnitude of the virus replication/spread. Although the oral exposure of animals leads to widespread virus dissemination and infection of a similar spectrum of tissues, animals remained asymptomatic following oral inoculation and also did not demonstrate abnormalities in hematologic parameters that have been associated with HCMV infection (Lockridge *et al.*, 1999). In contrast, animals inoculated intravenously developed a mononucleosis syndrome that was associated with monocytosis, thrombocytopenia, and leukocytosis (Lockridge *et al.*, 1999). These observations parallel clinical observations in human subjects infected inadvertently by blood transfusion or transplantation of an infected allograft. Because intravenous (or by an allograft) inoculation allows direct access to the circulatory system, there is likely no requirement for virus amplification in local

tissue prior to spread to visceral organs and regional lymphoid tissue. Thus, the virus can replicate to higher titers earlier in the course of the infection and presumably overwhelm the host innate immune responses. Clinical disease following acute CMV infection has been correlated with increased levels of replicating virus in humans as well as in experimental animal models of HCMV disease (Cope *et al.*, 1997; Spector *et al.*, 1998; Bernstein and Bourne, 1999; Kern, 1999; Emery *et al.*, 2000; Sequar *et al.*, 2002).

Following amplification in regional lymphoid tissue and the spleen and liver, dissemination of infectious virus results in the infection of sites that have been postulated to support persistent infection *in-vivo* and include salivary gland and breast secretory epithelium, prostatic epithelium, endometrium, and renal tubule epithelium (Becroft, 1981; Borisch *et al.*, 1988; Bale *et al.*, 1989; Sinzger *et al.*, 1995). Based on studies in experimental animal models and the findings that bone marrow and lung allografts can transmit infection, other sites such as the bone marrow and lung are also infected (Myerson *et al.*, 1984; Balthesen *et al.*, 1993; Kurz *et al.*, 1997; Salzberger *et al.*, 1997). The sites of long-term persistence, be it chronic productive infection or true latency, in humans are thought to include epithelium within exocrine glands, macrophage/monocytic cells, and hematopoietic stem cells. Pivotal aspects of the earliest phases of acute HCMV infection and dissemination remain inadequately studied. As an example, local amplification of virus following mucosal exposure has been postulated based on the kinetics of virus replication in the liver and spleen, yet there is little experimental evidence supporting this proposed mechanism. Alternatively, the virus could be transported to the regional lymph nodes, liver and spleen by circulating leucocytes prior to significant amplification in local tissue. The cell types that support local amplification are unknown and interactions between these cells and the innate immune system are not well described. The local host innate immune responses to naturally acquired HCMV infection could play a key role in the ultimate outcome of the infection, *i.e.* the level of virus replication and subsequent spread to the distant sites of infection. In contrast to the well documented role of the adaptive immune responses such as cytotoxic T lymphocytes and antiviral antibodies in the control and resolution of CMV infections, the importance of early innate responses at the site of inoculation to the course of infection with this virus remain poorly understood.

Transfusion and allograft-acquired infection

Infection with HCMV that follows exposure to blood products or an allograft more often resembles the symptomatic mononucleosis like syndrome that occurs in a minority of individuals who acquire HCMV by community exposure (Foster and Jack, 1969; Bowden, 1995). Presumably, development of symptomatic infection results from either the exposure to a large inoculum, delivery of infectious virus directly into the circulatory system, or by a combination of the two. In addition, allograft recipients as well as some recipients of blood products are often immunocompromised. Thus, the increase in HCMV burden is generally accelerated and the duration of active viral replication is increased secondary to the lack of normal immune responses to this virus. Clinical characteristics of this syndrome include fever, hematological abnormalities including leukocytosis often with monocytosis, and evidence of hepatocellular damage. Some patients will develop splenic enlargement and lymphadenopathy. Similar findings have been noted in animal models of acute HCMV infection (Bernstein and Bourne, 1999; Kern, 1999; Lockridge *et al.*, 1999). During peak virus replication, HCMV disseminates to end organs and sites of persistence as described above. Resolution of active virus replication is associated with control of clinical disease but virus excretion can continue for extended periods of time.

A consistent feature of HCMV infection in allograft recipients is the temporal sequence associated with virus replication and disease. In solid organ allograft recipients such as renal transplant recipients, virus replication and clinical symptoms are commonly observed several weeks following transplantation (Rubin and Colvin, 1986; Singh *et al.*, 1988; Rubin, 2002). The seemingly prolonged interval between transplantation and expression of an acute disease syndrome remains unexplained based on an expectation that virus reactivation/replication begins shortly after transplantation. It is possible that only vanishing amounts of virus are present in the allograft and replication of sufficient amounts of virus to induce disease requires this time interval. Consistent with this speculation is the finding that the virus burden in the blood closely parallels the development of disease in these patients and has been shown to increase exponentially in the time period preceding the development of disease (Cope *et al.*, 1997; Mendez *et al.*, 1998; Emery *et al.*, 2000; Nichols *et al.*, 2001). In contrast, disease in bone marrow allograft patients characteristically developed around 60 days post-transplantation and, based on these early studies in bone marrow allograft recipients, it was subsequently shown that detection of HCMV around day 35 post transplant in patients without clinical symptoms could be successfully treated with antiviral agents (Myers *et al.*, 1975; Schmidt *et al.*, 1991; Boeckh and Bowden, 1995; Boeckh *et al.*, 1997; Boeckh and Boivin, 1998; Nichols *et al.*, 2001). This finding is in agreement

with the general time frame of HCMV replication in solid organ transplant recipients and again suggests that HCMV must first establish a productive infection and amplify its genome copy number prior to dissemination to distant sites. Studies with murine CMV and immunocompromised mice have documented the importance of virus dissemination in the development of disease following reactivation of virus (Jonjic *et al.*, 1994; Reddehase *et al.*, 1994).

Spread within the host

Cell-associated spread within the host

Although it is has been suggested that cell free virus is responsible for community acquired HCMV infection, only limited data supports this claim. Perhaps the most convincing evidence comes from studies in breast feeding women that have demonstrated that infectious virus is present in the cell-free fraction of breast milk (Hamprecht *et al.*, 1998). This finding suggests that cell free virus can infect a mucosal surface. Animal models of CMV infection have utilized either intraperitoneal or subcutaneous inoculations almost exclusively; however oral inoculation with cell free murine CMV has been accomplished (personal communication, S. Jonjic, University of Rijeka, Rijeka, Croatia). In contrast to initial infection, the spread of HCMV within an infected host is likely to be cell associated based on findings from immuncompromised patients and studies in experimental animal models. In all but the most severely immunocompromised patients, infectivity that can be demonstrated in the blood compartment is most frequently associated with peripheral blood leukocytes and endothelial cells (Percivalle *et al.*, 1993; Waldman *et al.*, 1995; Salzberger *et al.*, 1997; Gerna *et al.*, 1998; Pooley *et al.*, 1999; Maidji *et al.*, 2002). The highest titers of infectious virus have been found associated with polymorphonuclear leukocytes from the buffy coat fraction of peripheral blood (Gerna *et al.*, 1992; Schafer *et al.*, 2000; Liapis *et al.*, 2003). Polymorphonuclear leukocytes cannot support virus replication but have been shown to carry infectious virus and viral gene products (Gerna *et al.*, 2000; Kas-Deelen *et al.*, 2001). In a series of in vitro experiments, Gerna and colleagues have demonstrated that HCMV infected endothelial cells or fibroblasts can transfer infectious virus to PMNs and in turn, these cells can transmit virus to susceptible fibroblasts (Gerna *et al.*, 2000). These studies have argued that microfusion events between virus containing vesicles and PMN are responsible for transmission of virus between cells (Gerna *et al.*, 2000). Although such a mechanism has not been experimentally verified in animal models of HCMV infection, the role of PMN in transmission of infectious HCMV in vivo is well accepted and the correlation between HCMV antigen positive PMN (antigenemia assay) and disseminated infection has been repeatedly shown to be a reliable diagnostic tool for the identification of patients at risk for invasive infection with HCMV (van der Bij *et al.*, 1988; The *et al.*, 1990; Gerna *et al.*, 1991; Erice *et al.*, 1992; Landry and Ferguson, 1993; Boeckh *et al.*, 1996; Nichols and Boeckh, 2000; Singh *et al.*, 2000). Interestingly, antigen positive PMN can be detected in normal hosts infected with HCMV but with a drastically reduced frequency as compared to immunocompromised patients, suggesting that even in normal hosts that PMN may be a common mode of virus dissemination. Other cells within the leukocyte fraction of peripheral blood cells support HCMV persistence and also transmit infectious virus. These include monocyte and macrophages derived by differentiation of blood monocytes (Rice *et al.*, 1984; Taylor-Wiedeman *et al.*, 1991; Fish *et al.*, 1995; Waldman *et al.*, 1995; Sinclair and Sissons, 1996; Guetta *et al.*, 1997; Soderberg-Naucler *et al.*, 1998; Hanson *et al.*, 1999; Jahn *et al.*, 1999; Riegler *et al.*, 2000). Cells derived from granulocyte/monocyte progenitor cells have been proposed as sites of latency based on in-vitro infections and can be detected as antigen containing cells in immunocompromised patients with disseminated HCMV infection (Taylor-Wiedeman *et al.*, 1991; Fish *et al.*, 1995; Kondo *et al.*, 1996; Sinclair and Sissons, 1996; Soderberg-Naucler *et al.*, 1997; Hahn *et al.*, 1998). In contrast, macrophages derived from peripheral blood of monocytes have been shown to harbor infectious HCMV upon stimulation with specific cytokines, including TNF-α (Taylor-Wiedeman *et al.*, 1994; Hummel *et al.*, 2001; Soderberg-Naucler *et al.*, 2001). Viral replication and expression of a variety of early and late proteins can be demonstrated in macrophages following infection with HCMV, although only recently derived clinical viral isolates efficiently infect these cells. This latter observation has been made independently by numerous laboratories and will be discussed in more detail in subsequent sections of this volume. The findings that individual strains of virus exhibited different biological behaviors in tissue culture have confirmed several observations that were first noted in the earliest in vitro studies of HCMV. Current studies are directed at deciphering the viral genes that account for these in-vitro phenotypes and may yield important new findings to help define the pathogenesis of HCMV infections.

A second cell lineage that is critical to the in-vivo spread of HCMV is the endothelial cell in a variety of microvascular beds in the human host. Endothelial cells have long been known to be a target for HCMV replication in vitro and infection of these cells results in a variety of cellular responses, including the release of cytokines and chemokines (Waldman *et al.*, 1991; Sinzger *et al.*, 1995,

1997; Waldman *et al.*, 1995; Plachter *et al.*, 1996; Fish *et al.*, 1998; Gerna *et al.*, 1998; Evans *et al.*, 1999; Kas-Deelen *et al.*, 2000; Brune *et al.*, 2001a,b; Maidji *et al.*, 2002; Odeberg *et al.*, 2002). Both lytic and non-lytic productive infections have been described in endothelial cells suggesting that depending on the source of cells and the infecting virus, endothelial cells can respond very differently to infection (Sinzger *et al.*, 1997, 2000; Fish *et al.*, 1998; Kahl *et al.*, 2000). Virus infection of endothelial cells is thought to be critical for infection of various tissues during HCMV dissemination and likewise, endothelial cell infection and virus release appears to be critical for the hematogenous spread from infected tissue (Waldman *et al.*, 1995; Gerna *et al.*, 2000; Maidji *et al.*, 2002). Early studies in transplant populations described viral antigen containing cells circulating in the blood of viremic transplant patients that subsequently were shown to be infected endothelial cells (Grefte *et al.*, 1993; Percivalle *et al.*, 1993; Gerna *et al.*, 1998). These cells contain infectious virus and are thought to represent infected endothelium that sloughs into the circulation, presumably secondary to local infection and/or inflammation. These cells are usually detected only in the most immunocompromised patients but these observations serve to illustrate that HCMV infection in these patients can be associated with an endothelitis that likely seeds the blood compartment with infectious virus containing cells. A similar role for endothelial cells in spread of CMV in both the murine model and guinea pig CMV model is assumed but has not been adequately explored (Brune *et al.*, 2001a,b).

Viral genes associated with virulence: viral dissemination and in vivo tropism

The large coding capacity of the HCMV genome in comparison to other herpes viruses has raised the possibility that a sizable number of these genes may encode functions that facilitate efficient in vivo replication, spread and persistence. This indeed appears to be the case as genetic comparisons between commonly used laboratory viruses and recent clinical isolates have demonstrated that laboratory isolates have large scale deletions yet replicate in vitro to levels that often exceed those of clinical isolates (Cha *et al.*, 1996). Studies utilizing laboratory isolates and recent clinical isolates have dramatic differences in tropism when cell types other than permissive human fibroblasts are used for in vitro propagation (Waldman *et al.*, 1991; Fish *et al.*, 1998; Soderberg-Naucler *et al.*, 1998; Jarvis *et al.*, 1999; Sinzger *et al.*, 1999, 2000; Bolovan-Fritts and Wiedeman, 2001; Brune *et al.*, 2001a,b; Gerna *et al.*, 2002). It is also almost certain that the phenotype of some viral genes that contribute to growth and persistence can only be defined in vivo and therefore will be extremely difficult to dissect in vitro. Studies in experimental animals, particularly the mouse have been quite revealing and point to the importance of viral genes in the initial replication and spread of CMVs. In studies investigating the spread of murine CMV from the site of inoculation to distant sites, infection has been was shown to be primarily associated with peripheral blood mononuclear cells. In these models the importance of an initial viremic spread to the liver and spleen was demonstrated and the role of peripheral blood mononuclear cell spread of virus to sites of persistence such as salivary glands was carefully documented (Bale and O'Neil, 1989; Collins *et al.*, 1994; Stoddart *et al.*, 1994; Mitchell *et al.*, 1996; Hanson *et al.*, 1999; Kern, 1999; Reddehase *et al.*, 2002). More recently, several reports have demonstrated the importance of individual viral genes in a murine model of CMV pathogenesis. At least three viral genes encoded by m139, 140, 141 orfs of MCMV have been shown to play a critical role in viral replication in monocyte/macrophages but have little to no effects on the replication of the virus in mouse fibroblasts (Saederup *et al.*, 1999; Hanson *et al.*, 2001; Saederup *et al.*, 2001; Menard *et al.*, 2003). The finding that viruses in which these genes have been deleted exhibit limited spread in vivo following intraperitoneal inoculation illustrates a potential role for these orfs in the in vivo pathogenesis of murine CMV (Saederup *et al.*, 2001; Mocarski, 2002). To date, the mechanism that accounts for restricted replication in monocytes of MCMV with deletions in these orf is unknown. The HCMV gene(s) that permit replication in monocyte/macrophages has not been definitively identified. Although it is far from clear if the observed expanded tropism of some strains of HCMV is required for the spread of HCMV in the infected host, the conservation of this phenotype amongst low passage clinical isolates and the subsequent loss of this phenotype upon passage in tissue culture would argue that monocyte/macrophage tropism contributes to the spread of the virus in the infected host. Genetic analysis of murine CMV indicated that endothelial cell tropism can be linked to a single viral gene, M45, the viral encoded ribonucleotide reductase and may be associated with resistance of endothelial cells to murine CMV induced apoptosis (Brune *et al.*, 2001a,b). The deletion of the homologous reading frame in HCMV was not associated with the loss of endothelial tropism (Hahn *et al.*, 2002). More recently, the importance of the VL129–131 of HCMV in endothelial tropism and presumably replication in vivo has been demonstrated by several laboratories (Hahn *et al.*, 2004; Wang and Shenk, 2005a,b). Thus, the viral genes and the mechanism by which products of the viral genes contribute to the expanded tropism of some clinical viral isolates of HCMV remain poorly understood.

Human cytomegalovirus encodes three G coupled protein receptor (GPCR) like molecules in orfs UL33, UL78,

US 27 and US28 (Margulies *et al.*, 1996; Streblow *et al.*, 1999; Rosenkilde *et al.*, 2001; Beisser *et al.*, 2002). The most extensively studied is US28, a GPCR that is constitutively activated and more importantly, can also signal after interaction with chemokines including RANTES, MCP-1, and fractalkine (Bodaghi *et al.*, 1998; Streblow *et al.*, 1999; Billstrom Schroeder and Worthen, 2001). Several laboratories have reported possible roles for this molecule in the spread of HCMV in vivo including, (i) acting as a chemokine sink to limit host cell chemotaxis to HCMV infected cells, (ii) providing an anti-apoptotic function, (iii) the recruitment of infected mononuclear cells to the sites of inflammation leading to dissemination of virus and (iv) perhaps even by binding of virus or virus infected cells to chemokine expressing endothelial cells based on observations of US28 binding membrane bound fractalkine (Bodaghi *et al.*, 1998; Kledal *et al.*, 1998; Billstrom Schroeder and Worthen, 2001; Beisser *et al.*, 2002; Billstrom Schroeder *et al.*, 2002; Randolph-Habecker *et al.*, 2002). In addition, infected smooth muscle cells expression US28 have been shown to migrate down chemokine gradients, thus providing an additional mechanism for the localization of HCMV infected cells to sites containing inflammatory cellular infiltrates (Streblow *et al.*, 1999). Although the role of US28 in HCMV induced vascular disease has been well studied and supported by in vitro models of smooth muscle cell migration, the importance of US28 in virus dissemination from local site of infection remains unclear. In murine CMV, two GPCR like gene products have been identified, M33 and M78 (Davis-Poynter *et al.*, 1997; Oliveira and Shenk, 2001; Waldhoer *et al.*, 2002). Studies have suggested that M33 is required for efficient virus dissemination to the salivary glands, a finding consistent with a potential role of US28 in the spread of HCMV (Davis-Poynter *et al.*, 1997). Recently, the US28 homologue of rhesus CMV has been identified (Penfold *et al.*, 2003). The phenotype of the M78 deletion virus suggests that the gene product of this orf is important in the replication of murine CMV in both monocytes/macrophages in vitro and in vivo (Oliveira and Shenk, 2001).

Another viral gene that appears to influence the spread of HCMV in vivo is UL146 (Penfold *et al.*, 1999). The protein encoded by this orf is a secreted protein that appears to function as a CXCL chemokine (*v*-CXCL1) that can induce chemotaxis and degranulation of PMNs (Penfold *et al.*, 1999). Interestingly this orf exhibits considerable sequence variability but maintains an amino terminal motif defining it as a CXCL chemokine (Penfold *et al.*, 1999). It is thought that this viral chemokine can recruit PMNs in vivo and thus could serve to disseminate HCMV from sites of infection. In severely immunocompromised hosts such as AIDs patients with gastrointestinal and retinal disease secondary to disseminated HCMV infection, neutrophil infiltration can be observed in the lamina propria as well as in the retina (Pepose *et al.*, 1985; Jacobson *et al.*, 1988; Francis *et al.*, 1989; Wilcox *et al.*, 1998). Infection of lamina propria macrophages with HCMV in vitro results in the induction of IL-8 release from these cells, suggesting that HCMV can both induce IL-8 release and encode a viral IL-8 like molecule (Redman *et al.*, 2002). Such findings are consistent with the proposed mechanism of chemokine expression and HCMV dissemination from sites of virus replication. Although this mechanism of dissemination is consistent with the histopathologic findings noted in severely immunocompromised patients, a neutrophil infiltrate is not an invariant feature of the histopathology of naturally acquired HCMV infections suggesting that interactions between this viral chemokine and other peripheral blood leukocytes that express its cognate cell surface receptor, CXCR2, also is required for virus dissemination. Alternatively, the recent findings that HCMV engages Toll-like receptors with resultant induction of pro-inflammatory cytokines and chemokines cascades suggests that the virus infection alone can recruit cells such as monocytes and PMN to sites of infection without the requirement of a specific viral chemokine (Compton *et al.*, 2003). Regardless of the specific mechanism and the role of a viral chemokine such as that encoded by orf 146, the intimate relationship between HCMV and components of the host inflammatory response likely represents a key step in the dissemination of HCMV.

As noted previously, studies in the murine model of CMV infection have provided data consistent with cell-associated virus spread (Bale and O'Neil, 1989; Collins *et al.*, 1994; Stoddart *et al.*, 1994; Mitchell *et al.*, 1996). Murine CMV encodes a related chemokine (orf m131), MCK-1, that initially was reported to function as a chemoattractant for monocyte/macrophages (Fleming *et al.*, 1999; Saederup *et al.*, 2001; Saederup and Mocarski, 2002). MCK-1 has been shown to induce calcium signaling and adherence of macrophages suggesting that it was a functional chemokine (Saederup *et al.*, 1999). In addition, these investigators suggested that its cellular receptor was the chemokine receptor, CCR3 (Saederup *et al.*, 1999). Later studies demonstrated that the major transcript and virus-expressed product of orf 131 was actually a spliced product in which the 5′ end of the 131 0RF was fused with the entire 129 orf to generate a spliced gene termed MCK-2 (MacDonald *et al.*, 1999). The protein product of MCK-2 shares a common chemokine domain encoded by m131 and thus is assumed to have similar activity as a chemokine (Mocarski, 2002). Perhaps the most interesting findings from this series of studies was that expression of the MCK-2 protein is non-essential for growth in fibroblasts

in vitro, but virus with deletions in this viral gene have a remarkable phenotype in vivo. The deletion of MCK-2 results in virus that cannot disseminate from local sites of infection and fails to disseminate in blood monocytes (Saederup *et al.*, 2001; Noda *et al.*, 2006). Histopathological studies demonstrated that the MCK-2 deletion virus did not induce the same degree of tissue inflammation and cellular (PMN) infiltrate as the wild type virus suggesting that this virus encoded chemokine facilitated spread from local sites of infection to distant sites of virus replication, presumably by recruiting monocytes and PMN to the initial sites of virus replication (Saederup *et al.*, 2001). Infection of these cells would then allow blood-borne dissemination of virus as infected leukocytes. This mechanism together with recent findings that envelope glycoproteins of HCMV can engage cellular Toll-like receptors (see above) and induce patterned responses of the innate immune system suggest that CMVs have subverted responses of the innate immune system to enhance their spread in vivo. Undoubtedly, additional mechanisms of virus dissemination will be uncovered as future studies unravel the function of the myriad on viral genes that are non-essential for in vitro virus replication in permissive cells. In addition, the development of more representative models of HCMV infection such as the rhesus macaque will also likely lead to a greater understanding of the role of various viral genes in the spread of HCMV in the infected host.

Disease and HCMV infection: pathogenesis of end-organ disease in acute infection

Disease associated with HCMV infection can be arbitrarily divided into manifestations that follow acute infection and diseases that appear to be associated with chronic infections. Considerably more is known about disease syndromes that follow acute infection because readily definable clinical abnormalities can be related to HCMV infection. In addition, disease can be temporally related to acquisition of the virus and often to levels of virus replication. Clinical disease has been most often related to the level of virus replication which in turn is dependent on characteristics of the host response, perhaps most importantly the host immune response. In severe infections, multiorgan involvement is often present and symptomatic disease is most commonly associated with some degree of organ system dysfunction or failure. The degree of organ dysfunction and overall disease has been most closely correlated with virus replication such that higher levels of virus replication are associated with more severe disease (Bowen *et al.*, 1996; Cope *et al.*, 1997; Baldanti *et al.*, 1998; Spector *et al.*, 1999; Emery *et al.*, 2000; Boppana *et al.*, 2001a,b; Nichols *et al.*, 2001; Boeckh *et al.*, 2003). In the absence of antiviral chemotherapy or the reconstitution of HCMV specific immune responsiveness, these patients often succumb to multiorgan failure. Studies in experimental animal models of HCMV disease, particularly those utilizing immunocompromised animals are consistent with many aspects of human disease, including the relationship between virus replication and disease (Kern, 1999; Lockridge *et al.*, 1999; Brune *et al.*, 2001a,b; Sequar *et al.*, 2002). When the data from numerous studies of HCMV infection in immunocompromised populations are examined, there appears to be no absolute level of viral burden that has been associated with disease. In fact, in these patients the relative increase in virus burden appears more predictive of disease. These findings would argue that the absolute level of virus replication as measured in body compartments such as the blood or urine merely indicates virus dissemination, whereas the rate of increase in virus replication (viral burden) could more accurately reflect the loss of immunological control of virus replication in infected organs and represents a harbinger of invasive disease and end-organ dysfunction. Results from studies in an experimental murine model of HCMV infections have indicated that monitoring the viral burden in the blood compartment only indirectly correlates with viral replication in infected organs and then only in immunocompromised animals (Brune *et al.*, 2001a,b). This experimental finding provides a possible explanation for the lack of a linear correlation between viral burden and disease and suggests that increasing viral burdens are of more diagnostic value in immunocompromised hosts and should be taken as evidence of continued virus replication and dissemination.

The histopathology of tissue from patients with acute HCMV syndromes suggests that organ dysfunction and disease is secondary to both direct viral cytopathogenic effects and indirectly through bystander damage secondary effector functions of the host as evidenced by the presence of inflammatory cells in histological tissue sections. The pathognomonic finding of HCMV infection in biopsy and autopsy specimens is the finding of so called owl-eye inclusions in large cells. These cells have been shown to be HCMV infected and their presence correlates with substantial HCMV replication (Mattes *et al.*, 2000). In experimental animal models of HCMV infection, increased viral copy number in target organs such as the liver, adrenal gland, and lung are associated with the level of tissue damage and the severity of disease (Brody and Craighead, 1974; Kern, 1999; Lockridge *et al.*, 1999). Similarly in humans with disseminated HCMV infections, virus-induced damage associated with histological evidence of HCMV infection can be demonstrated in the liver, adrenal glands, lung, pancreas, colon, esophagus, eyes and CNS (Bale *et al.*, 1989; Sinzger

and Jahn, 1996). Although virus-induced cytolysis and cellular necrosis could explain the disease manifestations of acute HCMV infection, additional mechanisms of cellular damage, including apoptosis have been suggested based on in vitro studies and in limited studies in experimental animal models (Kosugi *et al.*, 1998; Goldmacher *et al.*, 1999; Brune *et al.*, 2001a,b).

Early observations in bone marrow allograft recipients raised the possibility that HCMV pneumonitis in the post-transplant period was secondary to both the host immune response to HCMV and direct viral cytopathic effects (Grundy *et al.*, 1987; Barry *et al.*, 2000). Several lines of evidence are consistent with the hypothesis that the immune response to HCMV contribute to the pathogenesis of disease including; (i) the initial development of disease in the transplanted allograft, particularly in solid organ allograft recipients, (ii) the disparity between the limited distribution of virus infection in affected organs such as the lungs and the severity of clinical disease in patients with HCMV infection, (iii) the presence of inflammatory cellular infiltration and progressive disease in AIDs patients with invasive HCMV infections, (iv) the development of accelerated disease (immune vitritis) in AIDS patients with HCMV retinitis following reconstitution of antiviral adaptive immune responses (Karavellas *et al.*, 1998; Holland, 1999; Mutimer *et al.*, 2002). Together these data have argued that the host immune response represents at least one component of the pathogenesis of diseases associated with invasive HCMV infections. In contrast to the observations in AIDs patient following reconstitution of cellular immune responses, the control of virus replication by adaptive immunotherapy with HCMV specific CTL has been shown to decrease disease in allograft recipients (Walter *et al.*, 1995). This finding suggests that HCMV specific adaptive immune responses do not directly contribute to disease in the post-transplant period if present prior to widespread viral dissemination. Thus, it could be argued that the failure to restrict virus replication by more efficient host responses such as adaptive immunity could lead to end-organ damage secondary to the non-specific activities of cells of the innate immune system. In addition, the functional activity of virus-encoded cytokines, chemokine and chemokine receptors in HCMV disease in allograft rejection could also promote HCMV associated disease in allograft recipients by the recruitment of inflammatory myeloid cells into virus infected tissue Thus, the pathogenesis of disease observed during acute HCMV infection undoubtedly requires virus replication and expression of viral gene products, but also a significant but as yet unquantifiable contribution from the host immune response.

Although it can be argued that disease in immunocompromised hosts following acute infection with HCMV can be related to the level of virus replication, the variability of disease manifestations in different patient populations indicates that additional host factors contribute to the pathogenesis of this infection. As examples, the most common disease presentations in long-lived AIDs patients are retinitis and colitis (Blaser and Cohn, 1986; Pepose *et al.*, 1987; Francis *et al.*, 1989; Dieterich and Rahmin, 1991; Drew, 1992; Gallant *et al.*, 1992; Wilcox *et al.*, 1998; Pecorella *et al.*, 2000). Although HCMV colitis occurs in severely immunocompromised transplant patients, eye involvement is rare (Aldrete *et al.*, 1975; Kaplan *et al.*, 1989; Reed *et al.*, 1990). Similarly, in congenitally infected infants colitis is not a well described component of disseminated HCMV infection. Central nervous system involvement is a hallmark of congenital HCMV infections whereas it is rarely reported in transplant recipients and when present in AIDS patients, appears to be associated with similar CNS pathology and clinical features (Becroft, 1981; Morgello *et al.*, 1987; Wiley and Nelson, 1988; Schmidbauer *et al.*, 1989; Vinters *et al.*, 1989; Gallant *et al.*, 1992; Perlman and Argyle, 1992; Achim *et al.*, 1994; Arribas *et al.*, 1996). More recent studies of allograft recipients suggest that focal HCMU encephalitis may be more frequent that previously appreciated (Ribalta *et al.*, 2002). A variety of hypotheses have been put forth to account for the variability in disease between these groups of immunocompromised patients, yet none has adequately explained the differences in the manifestations of clinical disease. However, it is evident that virus dissemination and increased virus replication alone cannot account for the pathogenesis of acute HCMV infections. The balance between host innate and adaptive immune responses and the modulation of these responses by viral gene products is almost certainly central to our understanding of the pathogenesis of acute HCMV infection; however, it is also likely that other host responses unrelated to host immunity also play a significant role in the outcome of HCMV infection.

Disease and HCMV infection: pathogenesis of end-organ disease related to chronic infections

Although clinical syndromes associated with acute HCMV infections have received the bulk of experimental and clinical study, the role of the virus in chronic human disease has only recently become the subject of more intense study. As discussed previously, several laboratories have raised the possibility that HCMV infection was associated with a variety of human diseases including coronary atherosclerotic heart disease, gastric ulcer disease, rheumatologic disorders, and some human cancers. Note that each of these chronic diseases is also associated with inflammation, a host response that is also linked to HCMV replication and gene expression (Zhu *et al.*, 2002). Definitive evidence

demonstrating a role for HCMV in any of these diseases has not been reported, yet in the last decade a significant volume of observational data has linked HCMV infection with several of these diseases, including atherosclerotic vascular disease. In addition, studies in cardiac allograft recipients have reported the relationship between the development of transplant associated vascular sclerosis and HCMV infection (Grattan *et al.*, 1989; McDonald *et al.*, 1989; Loebe *et al.*, 1990; Everett *et al.*, 1992; Koskinen *et al.*, 1993; Hosenpud, 1999; Koskinen *et al.*, 1999; Streblow *et al.*, 2001). Aspects of many of these studies remain controversial but when viewed as a group, the data appear to support a role of HCMV in vascular disease (Table 41.5).

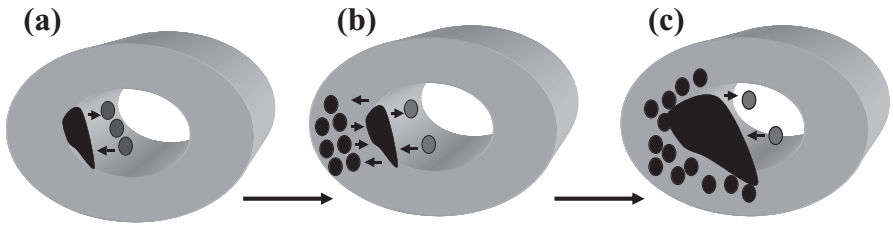
Perhaps more convincing support can be found in studies carried out in experimental animal models of allograft rejection and transplant associated vascular sclerosis. These studies have provided compelling evidence for the role of rodent CMVs in the development of post-transplant vasculopathy and coronary arteriosclerosis (Lemstrom *et al.*, 1995; Lemstrom *et al.*, 1997; De La Melena *et al.*, 2001). The relationship of HCMV infection to other chronic human diseases such as cancer is provocative yet far from conclusive (Huang and Pagano, 1978; Rapp and Robbins, 1984; Shen *et al.*, 1993; Cinatl *et al.*, 1996; Cobbs *et al.*, 2002; Harkins *et al.*, 2002). The impact of HCMV on the cell cycle during permissive infection and the less well understood relationship between viral gene expression during abortive infections and cell proliferation suggests that HCMV could at least facilitate the development of the malignant phenotype in some human cancers (Zhu *et al.*, 1995; Shen *et al.*, 1997; Fortunato *et al.*, 2000; Browne *et al.*, 2001a,b; Kalejta and Shenk, 2002; Kalejta *et al.*, 2003; Kalejta and Shenk, 2003). To unravel the possible roles of HCMV in human cancer, more comprehensive epidemiologic studies designed to determine possible relationships between infection with this ubiquitous virus and human cancer must be accomplished. In addition, it will be necessary to develop more informative in vitro model systems of cellular transformation following infection with HCMV.

In contrast to the relationship between the levels of virus replication and disease syndromes associated with acute HCMV infections, a similar relationship between HCMV virus replication and chronic diseases such as atherosclerosis in both normal and immunocompromised hosts has not been reported. To further complicate the study of HCMV infection and diseases such as coronary artery disease, it has been proposed that productive HCMV replication is not required for disease and that HCMV gene expression at a distant site could trigger disease in a target organ such as the heart (Zhou *et al.*, 1999). Finally, in vitro studies have also demonstrated that HCMV infection could be responsible for hit and run transformation event (Shen *et al.*, 1997). Thus, it is a daunting task to design a natural history study with sufficient power to identify a relationship between the development of a common disease such as coronary atherosclerotic heart disease or cancer and HCMV infection in populations in which the seroprevalence rates exceeds 60%–80%. Yet when the incredibly complex relationship between HCMV and the host cell and the life-long persistence of this virus in the host are considered together, it readily follows that HCMV could contribute to the pathogenesis of many of the diseases that have been only loosely associated with HCMV infection.

The inflammatory nature of many chronic diseases and the relationship between HCMV and inflammation has raised the possibility that HCMV is merely a passenger or bystander in these disease states and not causal. However, studies in transplant patients with accelerated vascular disease have provided strong support for a role of HCMV in chronic vascular disease observed in the normal host. The mechanisms by which HCMV promotes disease such as atherosclerosis or transplant vasculopathies are not completely understood but likely result from bi-directional interactions between HCMV and the host immune system. Studies from a number of laboratories have demonstrated that HCMV infection of host cells, including monocytes and endothelial cells, can induce expression of a variety of key mediators of the inflammatory response including adhesion molecules, chemokines, cytokines, and

Table 41.5. Evidence linking HCMV infection and vasculature disease. Studies that have demonstrated a link between HCMV infection and vascular disease

Study	Findings and interpretation
HCMV infection and coronary restenosis (Zhou *et al.*, 1996)	Increased rate of restenosis following coronary artery atherectomy in HCMV infected patients
HCMV antibody levels and carotid atherosclerosis (Nieto *et al.*, 1996)	Increased antibody titers correlated with more rapid onset of carotid vasculature disease
HCMV nucleic acids and antigen in coronary artery plaques (Wu *et al.*, 1992; Speir *et al.*, 1994)	HCMV directly associated with diseased vasculature
HCMV seropositivity and coronary artery disease (Melnick *et al.*, 1993; Epstein *et al.*, 1996; O'Connor *et al.*, 2001)	HCMV infection risk factor for development of coronary artery disease
HCMV infection and coronary vasculature disease in cardiac allograft recipients (Grattan *et al.*, 1989; Everett *et al.*, 1992; Hosenpud, 1999; Koskinen *et al.*, 1999; Valantine *et al.*, 2001)	Increased rate of vascular disease and graft loss in patients with HCMV infection



Vessel with developing plaque and/or focal area of damaged endothelium. (A) Inflammatory cellular infiltrate ( ) into area increasing local inflammation with additional damage to endothelium. (B) Release of chemokines and other attractants by mononuclear cells promotes migration of virus-infected smooth muscle cells ( ) into area. (C) Continued migration of smooth muscle cells into area leads to intimal thickening and narrowing of artery.

Fig. 41.1. Possible pathway of vasculopathy associated with HCMV infection of vessels.

pro-inflammatory host enzymes such as COX-2 (Van Dam-Mieras *et al.*, 1987; Taylor *et al.*, 1992; Koskinen, 1993; Sedmak *et al.*, 1994; Waldman and Knight, 1996; Yilmaz *et al.*, 1996; Craigen *et al.*, 1997; Grundy *et al.*, 1998; Speir *et al.*, 1998; Burns *et al.*, 1999; Redman *et al.*, 2002; Zhu *et al.*, 2002). In addition, HCMV encodes a number of immunomodulatory functions including chemokine receptors, chemokines (UL146), and cytokines (IL-10) as well as a plethora of viral evasion functions that favor viral persistence (Penfold *et al.*, 1999; Kotenko *et al.*, 2000; Spencer *et al.*, 2002). Together, interactions between virus infected cells and inflammatory cells could foster virus persistence while at the same time fueling chronic inflammation.

The proposed pathogenesis of HCMV associated vasculopathies in transplant patients is particularly revealing. Studies have reported that HCMV infection in the post-transplant period is a risk factor for graft dysfunction, including the development of vascular sclerosis in the transplant organ (Richardson *et al.*, 1981; Koskinen *et al.*, 1999; Browne *et al.*, 2001a,b; Soderberg-Naucler and Emery, 2001; Tolkoff-Rubin *et al.*, 2001). In cardiac allograft recipients, HCMV infection has been shown to be a risk factors for the development of a distinctive form of coronary arteriosclerosis in the transplanted heart (Grattan *et al.*, 1989; Koskinen *et al.*, 1994; Hosenpud, 1999; Koskinen *et al.*, 1999; Streblow *et al.*, 2003). A less than definitive clinical trial in cardiac allograft recipients with the antiviral agent ganciclovir suggested that inhibition of HCMV replication could limit the rate of development of vascular disease (Valantine *et al.*, 1999). Animal models of this disease process have been developed and data consistent with the observations in human disease have also been obtained (Lemstrom *et al.*, 1995; De La Melena *et al.*, 2001). In the rat model, rat CMV infection of animals with damaged endothelium is associated with infiltration of the intima with smooth muscle cells and narrowing of the lumen of coronary vessels of the transplanted heart (Lemstrom *et al.*, 1995; Vossen *et al.*, 1996; Li *et al.*, 1998; Zhou *et al.*, 1999; Streblow *et al.*, 2001). Early, but not late treatment of infected animals with antivirals can dramatically slow the development of these arterial lesions (Lemstrom *et al.*, 1997). From these experiments it appears that endothelial damage, inflammation and virus infection are required for the development of disease (Lemstrom *et al.*, 1995; Li *et al.*, 1998; Zhou *et al.*, 1999; Streblow *et al.*, 2003). Several models of the pathogenesis of this disease that combine these observations have been proposed. Some investigators have argued that CMV infection promotes systemic inflammation leading to an enhanced host inflammatory response that accelerates the development of vascular sclerosis in the transplanted organ. Other groups have argued that local expression of specific viral gene products in the target organ lead to disease in these animals. An example of such a viral gene product is the functional chemokine receptor HCMV US28. It has been shown that smooth muscle cells expressing the US28 viral gene product migrate down chemokine gradients (Streblow *et al.*, 1999; Streblow *et al.*, 2003). As illustrated in Fig. 41.1, damage to the endothelium either from virus infection, plaque deposition, or following allograft rejection in the case of cardiac allograft recipients, can lead to migration of infected smooth muscle cells expressing the viral chemokine receptor down a gradient of chemokines established by the infiltrating mononuclear cells or endothelial cells. The continued migration of these cells towards the gradient established by persistent local inflammation could account for the accumulation of these cell types in the intima of arteriosclerotic vessels. Because these virus infected smooth muscle cells also release inflammatory mediators, persistent HCMV infection could effectively recruit additional mononuclear cells into the focus of

infection. Infected cells, including activated mononuclear cells, can release inflammatory mediators that can further activate HCMV gene expression. Thus, infection with the virus establishes a paracrine loop that can utilize the products of host inflammation to drive viral gene expression and recruit uninfected and infected cells into an area of virus infection. Once a balance between virus replication and the elimination of virus infected cells is reached, HCMV can persist in the face of chronic host inflammation. Diseases such as coronary arteriosclerosis or transplant vasculopathy could then develop from a combination of HCMV infection and ongoing host inflammation. Although this pathway is consistent with observations made in experimental models of HCMV associated vascular disease, particularly in models of transplant vascular sclerosis, it remains to be shown if a similar pathway can be related to disease in humans. However, it is well accepted that chronic human diseases such as coronary artery disease have inflammation as a major component in their pathogenesis (Zhu *et al.*, 1999; Libby, 2002, 2003). Whether CMV plays a key role in chronic diseases such as coronary atherosclerosis remains unclear, yet this virus can be easily linked to chronic host inflammation and therefore remains a leading candidate as an infectious etiology of several chronic human diseases.

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Molecular basis of persistence and latency

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Introduction

Human cytomegalovirus (HCMV) is an ubiquitous β-herpesvirus that establishes a lifelong infection within the host. Although HCMV is generally asymptomatic within the normal individual, the virus causes severe and incapacitating disease in immune compromised patients (Pass, 2001). A critical component for HCMV persistence in the non-immune compromised host is the ability of the virus to establish cellular sites of latency as well as persistent infection. During latency, the HCMV genome is maintained within the cell with limited viral gene expression reactivating virus upon cellular stimulation. In HCMV persistent infection, infectious virus is continually produced in the cell with minimal cytopathic effect thereby enabling long-term infection. Endothelial cells (ECs) and specific subpopulations of the myeloid lineage are believed to represent important sites of persistent HCMV replication and latency, respectively. Recent studies of HCMV and the closely related murine cytomegalovirus (MCMV) are beginning to identify the virally encoded genetic determinants required for replication in these cell types. The establishment of latent infection in myeloid cells that are critical cellular components of the host immune system, also closely interconnects HCMV and the host immune response. This chapter will focus on the role of ECs and myeloid cells as sites of CMV persistent replication and latency, and the viral mechanisms that modulate cellular functions to ensure survival and reactivation within the host.

Sites of HCMV persistence and latency

HCMV infects many different cell types within the host, including: ECs, monocyte-derived macrophages (MDM), smooth muscle cells (SMCs), epithelial cells, fibroblasts, T-lymphocytes, granulocytes, stromal cells, neuronal cells and hepatocytes (Dankner *et al.*, 1990; Einhorn & Ost, 1984; Gnann *et al.*, 1985; Howell *et al.*, 1979; Myerson *et al.*, 1984; Schreier *et al.*, 1985; Sinzger *et al.*, 1995; Soderberg *et al.*, 1993; Wiley and Nelson, 1988). Recent studies have implicated ECs and specific cell types of the myeloid lineage [CD34+ hematopoietic progenitors, CD33+ granulocyte-macrophage progenitors (GM-Ps) and MDM] as critical sites of HCMV persistence and latency. The utilization of these sites as viral reservoirs appears to play an important role in enabling the virus to establish lifelong infection within the host. However, identification of these cell types as viral reservoirs does not preclude the existence of other sites of virus persistence within the host. For example, HCMV DNA has been identified in SMCs from the large arteries of healthy seropositive individuals (Gyorkey *et al.*, 1984; Hendrix *et al.*, 1989, 1990; Petrie *et al.*, 1987; Yamashiroya *et al.*, 1988). The absence of detectable viral gene expression in these HCMV DNA positive cells suggests that SMCs may represent a site of HCMV latency. The high incidence of HCMV transmission following bone marrow transplantation (BMT) (Ho, 1990) indicates that bone marrow stromal cells may also be a site of latency or persistent HCMV replication (Mayerson *et al.*, 1984). This observation is further supported by the ability to isolate infectious virus from the stroma of patients with HCMV disease, as well as the capacity to productively infect stromal cells with HCMV in vitro (Simmons *et al.*, 1990; Torok-Storb *et al.*, 1992). However, bone marrow stroma is composed of many different cell types, and ECs and myeloid progenitor cells within this heterogeneous population may actually represent the sites of virus persistence.

ECs are a site of persistent HCMV replication

ECs form the inner lining of blood vessels and are involved in a variety of processes regulating tissue homeostasis and

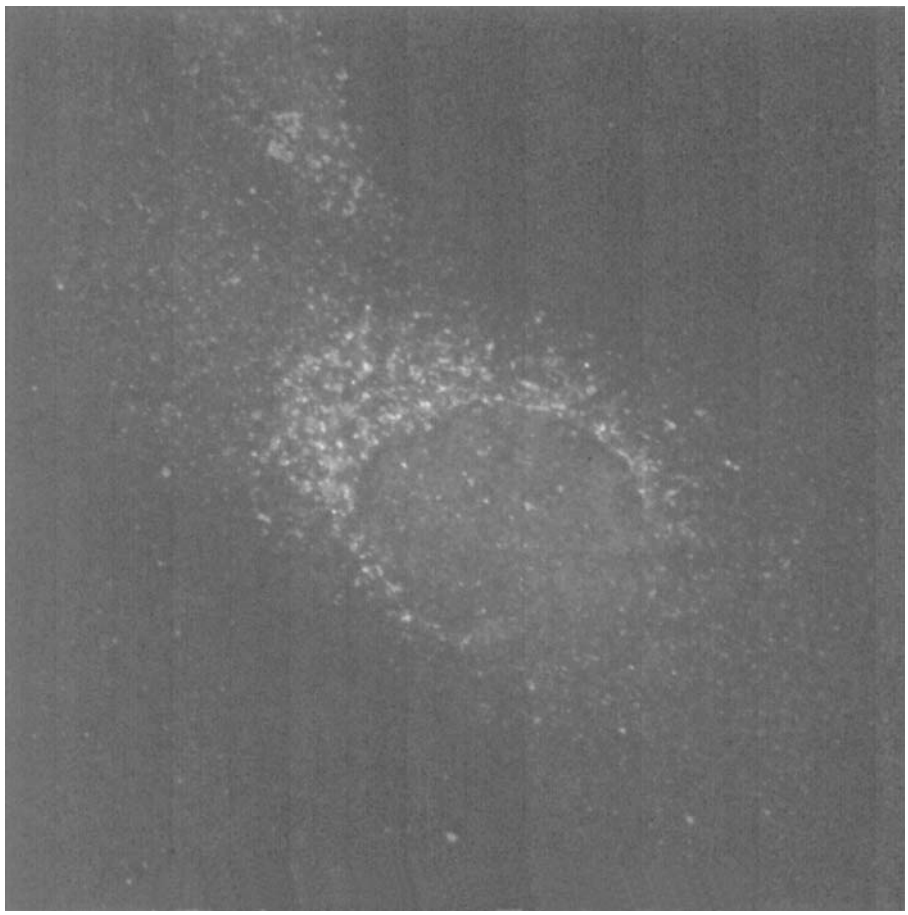

Fig. 42.1. Immunofluorescent micrograph of HCMV-infected AEC. Telomerase life-extended human AEC were infected with HCMV. Cells were fixed and stained for the presence of HCMV protein, glycoprotein B (a late product; green) and a cellular marker of the *trans*-Golgi network (TGN46; red). (See color plate section.)

inflammation. Results from a number of studies investigating the growth of HCMV in ECs have suggested a role for this cell type as an important reservoir of HCMV in the host (Fig. 42.1). The ability to infect cells with induction of minimal cytopathology is a critical requirement for a site of viral persistence. This virus-host cell interaction enables a persistent productive infection to be established for extended periods without cell death. Consistent with this requirement, early histological studies of BMT patients with acute HCMV disease showed that, in many cases, HCMV infection produced only minimal cytopathology in ECs (Friedman *et al.*, 1981). Subsequent studies identifying HCMV in the vessel walls of healthy individuals indicated that ECs also represented a site of persistent infection in individuals with normal immune function (Hendrix *et al.*, 1989; Pampou *et al.*, 2000; Smiley *et al.*, 1988; Speir *et al.*, 1994).

Recent studies have added considerably to our understanding of HCMV growth in ECs. ECs exhibit phenotypic differences that are dependent on the source (adult vs. fetal), vessel size (micro- vs. macrovascular) and anatomical location of the ECs (Page *et al.*, 1992; Turner *et al.*, 1987). Recent phage display-based assays using phage libraries expressing random peptides further emphasize the remarkable level of EC diversity, with ECs from different vascular beds expressing unique tissue-specific molecules (Rajotte *et al.*, 1998). EC origin also has a major influence on characteristics of HCMV replication. Studies comparing HCMV infection in human brain microvascular ECs (BMVEC) and macrovascular aortic ECs (AEC) have shown that HCMV replication differs significantly in these two cell types (Fish *et al.*, 1998). BMVEC, which together with astrocytes compose the blood brain barrier, possess specific transporter systems to allow the transfer of specific

metabolites from the blood to the brain parenchyma. Due to these specialized functional requirements, BMVEC are functionally and biochemically distinct from AEC (Joo, 1992; Moses & Nelson, 1994; Page *et al.*, 1992; Turner *et al.*, 1987). Although both BMVEC and AEC express viral proteins and support HCMV replication, virus fails to accumulate intracellularly in AEC resulting in a reduced level of cell-associated compared to supernatant virus (Fish *et al.*, 1998). In contrast, virus accumulates intracellularly in BMVEC resulting in comparable levels of cell-associated and supernatant virus. This difference in the distribution of virus corresponds to HCMV infection resulting in a lytic infection in BMVEC, but not AEC, suggesting that efficient removal of mature intracellular virions (by either export or degradation) may enable prolonged cell survival. Importantly, this ability of HCMV to produce a persistent long-term productive infection in AEC suggests that AEC may represent a site of persistence within the host.

Differences between individual HCMV strains also have a profound influence on HCMV growth in ECs. Results from a number of laboratories suggest that EC and non-EC tropic HCMV strains are comparable in their ability to enter ECs. However, non-EC strains appear to be deficient in the ability to translocate the viral genome to the nucleus (Bolovan-Fritts and Wiedeman, 2001; Sinzger *et al.*, 2000; Slobbe-van Drunen *et al.*, 1998). Interpretation of results from these studies has been complicated by observed differences in the capacity of even identical strains of HCMV to replicate in ECs (Bolovan-Fritts and Wiedeman, 2001; Kahl *et al.*, 2000). These discrepancies may result from differences in virus preparation and EC culture or HCMV strain derivation. For example, when compared to the United Kingdom (UK) strain, the same HCMV laboratory strain from the American Tissue Culture Collection (ATCC) was shown to differ by the presence of a 929-bp genomic region (Dargan *et al.*, 1997; Mocarski *et al.*, 1997). The cloning of an increasing number of HCMV strains as genetically stable bacterial artificial chromosomes (BACs) is overcoming many of these technical problems and is beginning to facilitate the identification of genetic determinants of HCMV EC tropism (Borst *et al.*, 1999; Hahn *et al.*, 2002, 2003; Marchini *et al.*, 2001; Yu *et al.*, 2002).

The endothelium represents the cellular interface between the blood and underlying tissues. A number of studies indicate that HCMV may facilitate hematogenous spread of virus by modulating interactions between ECs and monocytes (an important site of latent HCMV infection; see below) (Cebulla *et al.*, 2000; Knight *et al.*, 1999; Waldman *et al.*, 1995). HCMV infection of ECs increases expression of cell adhesion molecules such as ICAM-1, which corresponds to an increased interaction between ECs and monocytes resulting in monocyte infection (Knight *et al.*, 1999). Monocytes infected in this manner were capable of transmitting virus to uninfected ECs suggesting a possible mechanism for HCMV dissemination in vivo (Waldman *et al.*, 1995). ICAM-1 was shown to be upregulated at the transcriptional level by the interaction of two HCMV transcriptional activators (IE1 and IE2) with the ICAM-1 promoter (Burns *et al.*, 1999). HCMV infection also upregulates adhesion molecules indirectly by the induction of proinflammatory cytokines. HCMV-infected ECs have been shown to induce vigorous lymphocyte proliferative responses in allogeneic HCMV seropositive-derived T cells resulting in the release of IFN-γ and TNF-α (Waldman and Knight, 1996). The ability of HCMV to induce TNF-α expression in monocytes may play a critical role in disease progression due to the role of this cytokine in HCMV reactivation from this cell type (see below). These proinflammatory cytokines were also shown to induce expression of immune response molecules (ICAM-1, VCAM-1, MHC-I and MHC-II) in neighboring uninfected EC (Waldman and Knight, 1996). Clinically, CMV-mediated upregulation of adhesion and immune response molecules within transplants of solid organ transplant (SOT) and BMT patients may also increase the immunogenicity of the graft resulting in increased incidence of graft rejection (a serious complication of transplantation closely associated with HCMV infection) (Girgis *et al.*, 1996; Grattan *et al.*, 1989; Humar *et al.*, 1999a; Keenan *et al.*, 1991; Koskinen *et al.*, 1996; Smith *et al.*, 1998).

Determinants of EC tropism

The dependence of HCMV replication in ECs on the virus strain suggests that specific viral genes are required for efficient replication in this cell type (Kahl *et al.*, 2000; MacCormac and Grundy, 1999; Sinzger *et al.*, 1999). However, until the recent ability to clone CMV genomes as BACs (for review see: (Wagner *et al.*, 2002)), the instability of EC tropism during in vitro culture of HCMV posed a major problem to the identification of EC tropism determinants. Although, most low passage clinical isolates can initially replicate in both ECs and fibroblasts, these isolates consistently lose their ability to replicate in ECs following repeated passage in fibroblasts (MacCormac and Grundy, 1999; Sinzger *et al.*, 1999). However, initial plaque purification of EC tropic strains and isolation of single clones results in stable maintenance of EC tropism (even after long-term passage in fibroblasts). This result suggests that loss of EC tropism results from selection of viral variants within the initial clinical isolate rather than from viral mutants derived by *de novo* mutation or from non-genetic alterations of the virus (Sinzger *et al.*, 1999). The presence of multiple

genetic determinants directing EC tropism is suggested by the observation that recent clinical isolates exhibit a gradation in their ability to replicate in ECs (Sinzger *et al.*, 1999). Alternatively, this observation could be explained by differences in expression level of a single gene or the existence of polymorphic forms of a single genetic determinant. However, the existence of multiple genes for EC tropism is further supported by the observation that co-infection with two distinct non-EC tropic strains resulted in production of a genetically stable EC-tropic recombinant virus (Sinzger *et al.*, 1999). This result could be explained by two mutations present in the same gene having been repaired by a recombination event. Alternatively, a more likely explanation is one of recombination relocating two distinct genetic loci required for EC tropism within the same recombinant genome.

More recently, BAC-based deletional mutagenesis studies have identified a "genomic tropism island" composed of three HCMV ORFs: UL128, UL130 and UL131A that is required for replication in ECs (Hahn *et al.*, 2004). Deletional mutagenesis identified UL128, UL130 and UL131A as each individually required for replication in human umbilical vein ECs (HUVECs). The inability of a number of laboratory strains to replicate in ECs was also consistent with these viruses encoding inactivated forms of UL128, UL130 and UL131A. Heterologous expression of products of each these ORFs recovered EC tropism of viruses that expressed inactivated versions of the respective ORF, identifying the product of each ORF as individually required for EC tropism. The pUL130 and pUL128 proteins are components of the virion envelope (Patrone *et al.*, 2005; Wang and Shenk, 2005b) that form a complex with two essential envelope glycoproteins (gH and gL) (Wang and Shenk, 2005b). The gH and gL glycoproteins had previously been known to complex with a third glycoprotein, gO, and to be required for replication in fibroblasts (Hobom *et al.*, 2000). Two distinct complexes have been identified in EC-tropic virions comprised of gH/gL complexed with either pUL128/pUL130 or gO (Wang and Shenk, 2005b). Antibodies directed against either pUL130 and pUL128 inhibit infection of ECs (HUVECs), but not fibroblasts. Together, these results support a model wherein the pUL128/pUL130 and the gO containing gH/gl complex are required for infection of ECs and fibroblasts, respectively. The pUL131A has not been detected in the gH/gl complex with pUL128 and pUL130. However, a functional UL131A ORF is required for incorporation of pUL128 and pUL130 into the gH/gl virion associated complex (Wang and Shenk, 2005b), and UL131A is required for an early stage of the virus replication cycle in ECs (Wang and Shenk, 2005a). These observations suggest that UL131A is also probably involved in mediating virus entry into ECs, but may be required at submolar levels, or perhaps is more weakly associated with the complex. UL131A is also required for HCMV replication in lung microvascular ECs and a variety of epithelial cell types (Wang and Shenk, 2005a), as well as monocyte-derived dendritic cells (GM-CSF and IL-4 derived) (Gerna *et al.*, 2005). The requirement of these ORFs for replication in other biologically relevant types of ECs as well as MDM is unknown.

Studies in the closely related MCMV model have also identified a number of viral genes required for growth in ECs as well as macrophages (see below). These studies have similarly relied heavily on BAC-based technology and further emphasize the strength of bacterial-based genetic approaches to address these questions. A recent forward genetic approach identified the MCMV encoded ribonucleotide reductase R1 subunit homologue, M45, as necessary for MCMV growth in murine ECs in vitro (Brune *et al.*, 2001). M45 was also required for normal in vitro replication in macrophages, but not fibroblasts, bone marrow stromal cells or hepatocytes. ECs infected with M45 deletion mutants died rapidly from apoptosis indicating that this gene enabled MCMV replication in ECs by preventing apoptosis of the infected cell. Since ECs represent a site of persistent virus infection, the ability to prevent the normal apoptotic death response of these cells to viral infection may be crucial for CMV replication in this cell type. However, a subsequent study showed that the HCMV M45 homologue (UL45) is not required for growth of a BAC cloned recent clinical isolate (RVFIX) in ECs (HUVEC) indicating that homologous genes in HCMV and MCMV may not always be functionally interchangeable (Hahn *et al.*, 2002). Alternatively, given the heterogeneity of ECs, the function of UL45 during infection of HUVEC may not accurately reflect the role of this gene during HCMV replication in EC types normally infected in vivo. The specific level within the apoptotic pathway that the M45 product functions is unknown. A study from our laboratory investigating determinants of rhesus cytomegalovirus (RhCMV) cellular tropism has identified a virally encoded cyclooxygenase 2 (vCOX-2; Rh10) as a critical determinant of RhCMV EC tropism (Rue *et al.*, 2004). The vCOX-2 appears to mediate EC tropism by inhibiting production of the cellular antiviral molecule, nitric oxide (NO). Although HCMV does not encode a COX-2 homologue, the virus is known to induce cellular COX-2 expression, suggesting that COX-2-mediated inhibition of NO synthesis may be an important determinant for EC tropism.

Myeloid lineage cells are a site of HCMV latency

In addition to ECs as a site of HCMV persistent infection, specific subpopulations of cells of the myeloid lineage have

been identified as potential sites of HCMV latency. Within the periphery, CD14+ monocytes are a known site of latent HCMV infection. The ability to reactivate virus from naturally infected CD14+ monocytes by differentiation into MDM in vitro definitively identifies these cells as a site of latent HCMV infection. Within the bone marrow, CD34+ hematopoietic progenitors and GM-Ps have also been identified as potential sites of HCMV latency. However, reactivation of HCMV from these cell types has been observed only following *in vitro* infection. Consequently, the role of these cell types as sites of HCMV latency in vivo remains unclear.

CD14+ monocytes and MDM

The high level of HCMV transmission associated with the transfusion of blood products (Bowden, 1995; Bowden *et al.*, 1995; Hersman *et al.*, 1982; Tegtmeier, 1989), and specifically with cells of the leukocyte fraction, was the initial indication that leukocytes represented a significant source of latent or persistent virus (Adler *et al.*, 1983, 1983; Tegtmeier, 1986). Subsequently, PCR analysis for HCMV DNA identified CD14+ monocytes as the predominant HCMV-infected cell type in the blood of normal immune competent HCMV seropositive individuals (Taylor-Wiedeman *et al.*, 1991, 1993). PCR analysis showed that only a small number of monocytes in the peripheral blood contained HCMV DNA (<1 in 10^4) (Slobedman and Mocarski, 1999), and viral gene expression in these cells was shown either to be undetectable or restricted to immediate-early gene expression (Dankner *et al.*, 1990; Taylor-Wiedeman, *et al.*, 1991, 1993). These in vivo infection results were consistent with findings from in vitro studies, wherein HCMV infection of monocytes was inefficient, with viral replication being absent or restricted to early events of gene expression (Einhorn and Ost, 1984; Rice *et al.*, 1984; Söderberg *et al.*, 1993). Importantly, the ability of the leukocyte fraction to transmit infection, combined with the absence of viral gene expression in the major infected cell type within this population (CD14+ monocytes), identified CD14+ monocytes as a potential site of viral latency. Consistent with monocytes representing a site of HCMV latency, the HCMV genome in these cells was shown to be maintained at a relatively low copy number (6–13 copies/cell) (Slobedman and Mocarski, 1999) and in an episomal circular form (Bolovan-Fritts *et al.*, 1999) comparable to the latent genome structure of other herpesviruses.

Definitive evidence identifying CD14+ monocytes as a site of HCMV latency requires demonstration of an ability to reactivate virus from naturally infected cells. However, initial attempts to reactivate virus from naturally infected monocytes (Ibanez *et al.*, 1991; Taylor-Wiedeman *et al.*, 1994), or even to establish productive replication following in vitro infection were unsuccessful (Einhorn and Ost, 1984; Rice *et al.*, 1984; Söderberg *et al.*, 1993). In one study, productive HCMV replication following in vitro infection was detected, but infection was extremely inefficient with levels of virus progeny at 0.1% of the level observed in fibroblasts (Maciejewski *et al.*, 1993). A major break-through came with a series of experiments that revealed the importance of monocyte/MDM differentiation-state for HCMV replication (Fig. 42.2) (Ibanez *et al.*, 1991). Specifically, differentiation of monocytes into MDM by co-culture with concanavalin A (Con A)-stimulated non-adherent cells dramatically increased the ability of HCMV to replicate in these cells with approximately 40% of MDM (designated Con A-MDM) supporting productive HCMV replication following in vitro infection (Ibanez *et al.*, 1991). Subsequent studies demonstrated that differentiation of naturally infected peripheral CD14+ monocytes into MDM (designated Allo-MDM) by allogeneic stimulation with non-adherent peripheral blood mononuclear cells (PBMC) was able to reactivate virus from these cells (Söderberg-Naucler *et al.*, 1997b). This report was the first study to conclusively identify myeloid lineage cells as a reservoir for HCMV latency, and has enabled the study of mechanisms of reactivation of latent virus from naturally infected cells.

Growth of HCMV in Con A-MDM

The initial studies using cocultivation with Con A-stimulated autologous non-adherent PBMCs to induce monocyte differentiation resulted in production of the Con A-MDM phenotype that was permissive to HCMV, and resulted in a nonlytic infection that produced exclusively cell-associated virus (Ibanez *et al.*, 1991; Söderberg-Naucler *et al.*, 1997a). Generation of this Con A-MDM phenotype was shown to require CD8+ T-lymphocytes and the proinflammatory cytokines IFN-γ and TNF-α (but not IL-1, IL-2, TGF-β or GM-CSF) (Söderberg-Naucler *et al.*, 1997a). Addition of recombinant IFN-γ or TNF-α alone to monocyte cultures was sufficient to produce MDM that were comparable to Con A-MDM in their level of virus production (Söderberg-Naucler *et al.*, 1997a). These observations suggest a model wherein production of IFN-γ and TNF-α by Con A-activated CD8+ T-lymphocytes induces monocyte differentiation into HCMV permissive Con A-MDM (Fig. 42.3), and indicate a critical role for immune stimulation in the production of HCMV-permissive MDM. Interestingly, IFN-γ and TNF-α were also shown to reactivate HCMV from latently in vitro infected GM-Ps (Hahn *et al.*, 1998) identifying a common requirement for these cytokines in the production of an HCMV-permissive cellular phenotype. A more direct role of TNF-α in HCMV

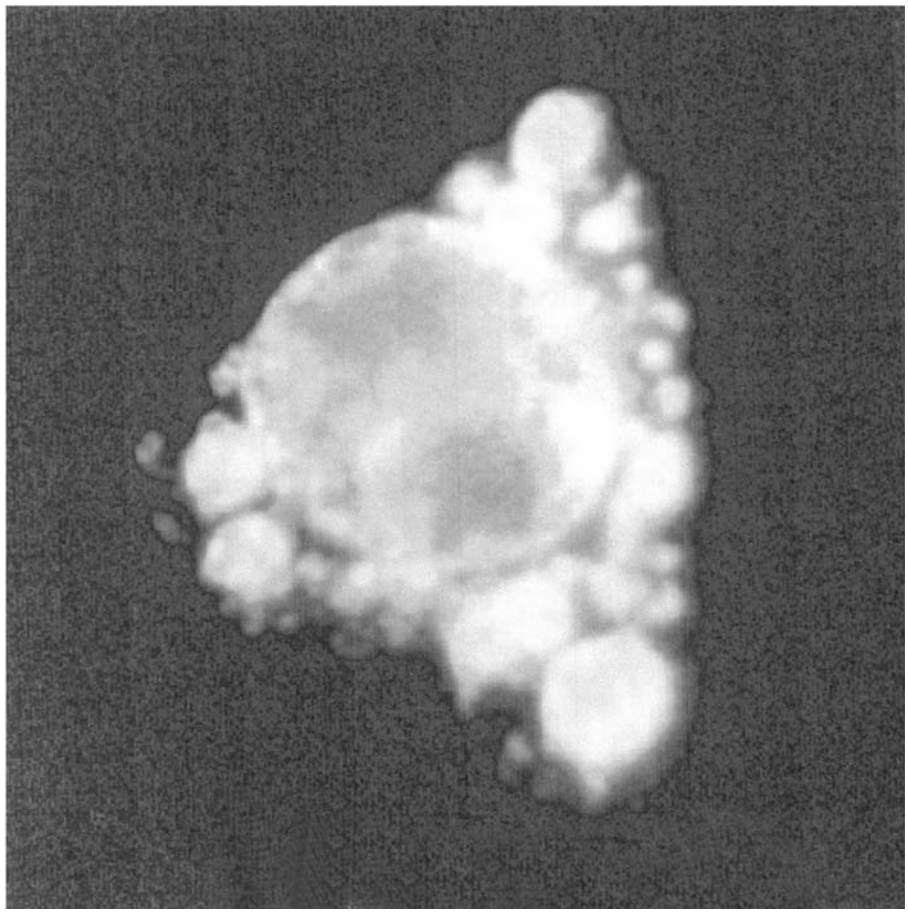

Fig. 42.2. Immunofluorescent micrograph of HCMV-infected MDM. MDM were infected with HCMV. Cells were fixed and stained for the presence of HCMV proteins, pp65 (an early late product; green) and IE-2 (an immediate-early product; red). (See color plate section.)

reactivation is also suggested by the ability of this cytokine to stimulate the IE promoter in myeloid cells by activation of NFκB. Activated NFκB translocates to the nucleus and binds directly to NFκB binding motifs in the IE enhancer thereby activating expression of the two main transcriptional activators of the virus (Prösch *et al.*, 1995, 2001; Ritter *et al.*, 2000; Stein *et al.*, 1993; Zhang *et al.*, 2001). The Con A-MDM model was a major advance in understanding mechanisms of HCMV reactivation. However, the inability to reactivate HCMV from naturally infected Con A-MDM prevented the definitive identification of CD14+ monocytes as a site of HCMV latency in vivo.

Growth of HCMV in Allo-MDM

Allogeneically stimulated PBMC have recently been used to induce MDM differentiation in a cellular microenvironment more closely resembling the milieu believed to exist during in vivo reactivation of HCMV from monocytes. In this system, coculture of HLA-mismatched donor PBMC populations resulted in the production of MDM (designated Allo-MDM) that were permissive to HCMV infection and enabled reactivation of latent virus from CD14+ monocytes naturally infected in vivo (Söderberg-Naucler *et al.*, 1997b). Allo-MDM express both macrophage (CD14/CD64) and dendritic (CD1a/CD83) cell markers. In contrast to Con A-MDM, in vitro infection of Allo-MDM is characterized by vigorous lytic replication in a large number of cells (>50%) and results in accumulation of extracellular virus (Söderberg-Naucler *et al.*, 2001). Importantly, latent HCMV can be reactivated from naturally infected Allo-MDM, which is not possible using other MDM culture systems (Söderberg-Naucler *et al.*, 1997a, b, 2001) and emphasizes the importance of the specific MDM differentiation pathway for HCMV reactivation.

In the Allo-MDM system, cellular depletion and cytokine neutralization experiments showed that both CD4+ and

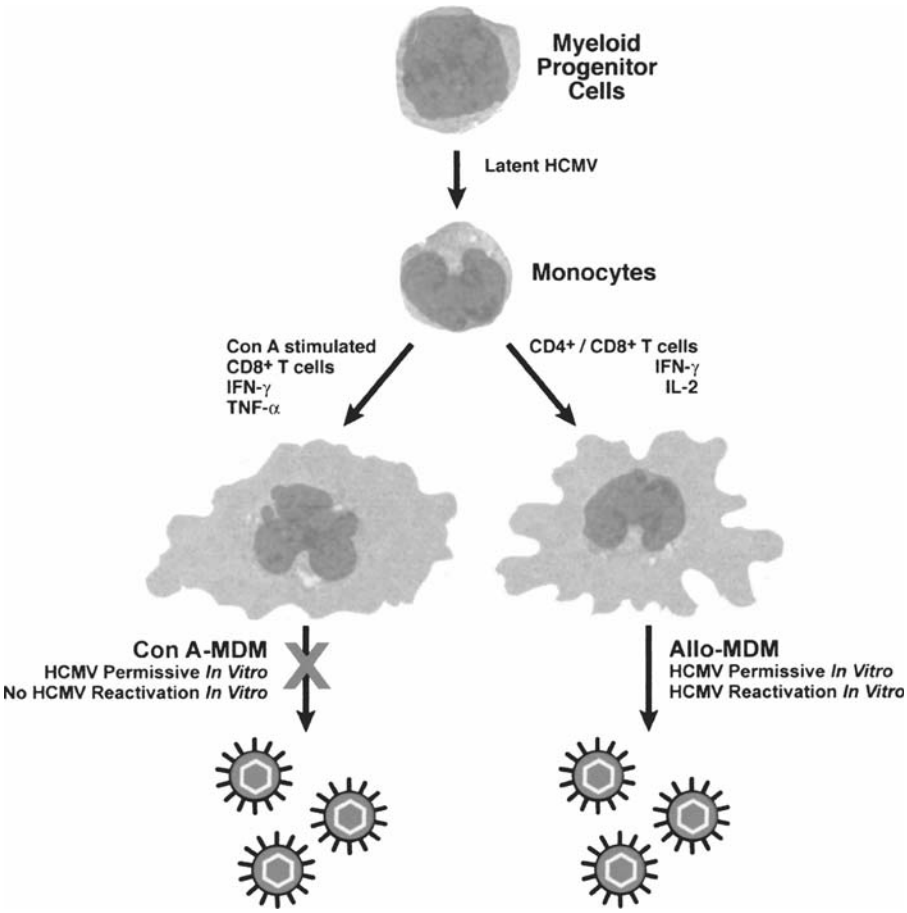

Fig. 42.3. Schematic showing cellular and cytokine factors necessary for generation of Con A- and Allo-MDM.

CD8+ T lymphocytes and the cytokines IFN-γ and IL-2 (but not IL-1, TNF-α or TGF-β) were required for generation of the HCMV-permissive phenotype (Fig. 42.3) (Söderberg-Naucler *et al.*, 2001). These cytokines were required for induction of an Allo-MDM phenotype able to support HCMV replication following in vitro infection. However, reactivation of latent virus from naturally infected cells required additional factors. Conditioned media from Allo-MDM cultures stimulated reactivation of HCMV in the absence of allogeneic stimulation. Thus, soluble factors released during allogeneic stimulation are sufficient to produce a MDM phenotype capable of HCMV reactivation. The presence of IFN-γ (but not IL-1, IL-2, TNF-α, TGF-β or GM-CSF) within the first 48 hours of allogeneic stimulation was necessary for efficient reactivation of latent HCMV. However, IFN-γ alone was not sufficient for the induction of a MDM phenotype capable of reactivating virus. Analysis of the cytokines released during Allo- compared to Con A-MDM differentiation has revealed considerable differences in the kinetics as well as in the type of cytokines released by the two differentiation pathways. IL-13 was observed only in Allo-MDM cultures. IL-13 has previously been shown to increase, albeit at low levels, HCMV gene expression and replication in in vitro infected alveolar macrophages and may be important in the reactivation of HCMV from latency (Hatch *et al.*, 1997). These studies suggest a model (Fig. 42.4) wherein latently infected CD14+ monocytes in the peripheral blood are activated by an immune response (ie., allogeneic organ transplantation or blood transfusion). During this activation, CD4+ and CD8+ lymphocytes play a critical role, with IL-2 released from CD4+ lymphocytes inducing the release of soluble factors from CD8+ lymphocytes. These soluble factors, which include IFN-γ, induce differentiation of CD14+ monocytes into the Allo-MDM phenotype required for reactivation of latent virus.

Consistent with the Allo-MDM in vitro model, a number of studies support a role for immune activation accompanied by release of proinflammatory cytokines

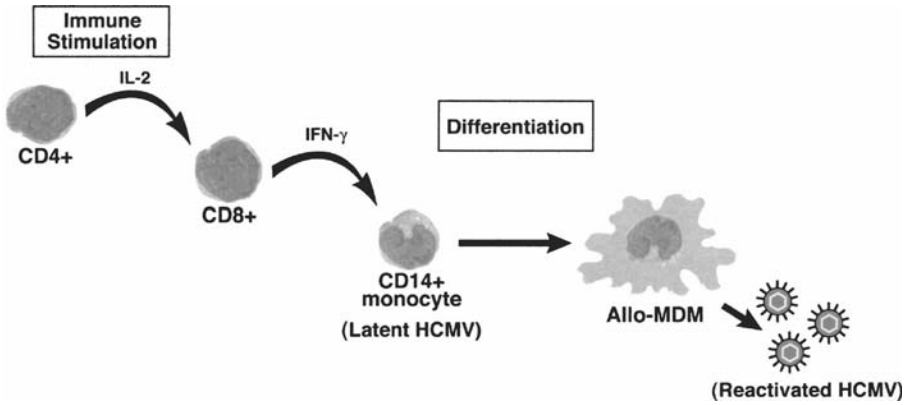

Fig. 42.4. Model for reactivation of latent HCMV in vivo. Allogeneic reaction during transplantation or blood transfusion results in IL-2 production by CD4+ T cells with subsequent stimulation of IFN-γ secretion by CD8+ T cells. In concert with other factors, IFN-γ induces CD14+ monocyte differentiation into Allo-MDM, reactivating latent virus and resulting in the production of infectious virus.

in HCMV reactivation and replication in vivo. In SOT patients, the recipient immune system launches a vigorous allogeneic immune response within the graft in an attempt to remove the transplanted tissue. This response is associated with the release of proinflammatory cytokines, chemokines and an increased expression of adhesion molecules. High levels of HCMV reactivation and disease are consistently observed in allogeneic graft tissue, and elevated plasma levels of IFN-γ and TNF-α are found in patients with HCMV disease (Docke *et al.*, 1994; Fietze *et al.*, 1994; Humar *et al.*, 1999a,b). This association of HCMV disease with proinflammatory cytokine levels is also observed in SOT patients receiving anti-lymphocyte γ globulin (ALG). In these cases, increased TNF-α levels associated with ALG treatment correspond to an increase in severity of HCMV disease (Tolkoff-Rubin and Rubin, 1994). The clinical significance of this extensive reactivation and induction of viral replication within the graft is illustrated by the high incidence of HCMV cytopathology and disease within the transplanted organ (Ho, 1990). For example, HCMV hepatitis is normally asymptomatic in non-hepatic SOT patients; however, in liver transplant recipients, HCMV hepatitis is frequently observed. Similarly, in heart–lung transplant recipients the incidence of HCMV pneumonia is significantly elevated as compared to other SOT patients (Ho, 1990). Since blood transfusion is commonly performed over histocompatibility barriers, reactivation of HCMV through allogeneic stimulation of PBMC would also be expected to play a role in HCMV transmission following transfusion. The high levels of HCMV antigenemia observed in patients with chronic plaque psoriasis are also consistent with the increased TNF-α expression observed in these patients (Asadullah *et al.*, 1999). In experimental studies of MCMV, allogeneic transplantation induces expression of CMV IE genes in vivo, which is accompanied by an increased expression of transcripts for IL-2, TNF-α, IFN-γ and activation of NFκB (Hummel *et al.*, 2001). Although the ability to reactivate productive virus was not determined, a separate study showed that MCMV IE expression could be induced in the lung of latently infected mice in vivo by treatment with TNF-α without immune suppression (Koffron *et al.*, 1999).

Other inflammatory cytokines and chemokines have also been associated with increased HCMV replication and disease following transplantation (Nordoy *et al.*, 1999; 2000a, b). IL-8 was shown to increase HCMV replication in MRC-5 fibroblasts (Murayama *et al.*, 1998), and may be of particular importance due to the high levels of IL-8 that are secreted from a variety of cells in response to proinflammatory cytokines released from allogeneically activated MDM within the graft (Harada *et al.*, 1994). HCMV has also been shown to induce IL-8 expression (Murayama *et al.*, 1998), and studies suggest that IL-8 may function by inhibition of the antiviral IFN-γ response pathway or by increasing neutrophil-mediated HCMV dissemination by attracting neutrophils to sites of HCMV replication (Grundy *et al.*, 1998). High levels of the anti-inflammatory cytokine IL-10 have been shown to be associated with HCMV disease in renal SOT recipients (Nordoy *et al.*, 2000b). An HCMV encoded IL-10 homologue (cmvIL-10) encoded by ORF UL111a was recently identified (Kotenko *et al.*, 2000). The cmvIL-10 shares low sequence homology (27%) with cellular IL-10; however, cmvIL-10 binds to the cellular IL-10 receptor and competes with cellular IL-10 for binding to this receptor. The biological activity

of cmvIL-10 and cellular IL10 also appear comparable (Spencer *et al.*, 2002). Paradoxically, IL-10 has been shown to inhibit monocyte and macrophage function, including inhibiting the release of TNF-α from activated MDM (Oswald *et al.*, 1992). However, recent studies indicate that IL-10 is involved in macrophage differentiation (Allavena *et al.*, 1998), and therefore high IL-10 levels may induce differentiation of MDM into a phenotype that is permissive for HCMV. Together, results from these studies suggest that immune activation with concomitant production of proinflammatory cytokines plays a critical role in virus reactivation and replication in vivo in naturally infected patients, which is recapitulated in vitro in the Allo-MDM system.

CD34+ hematopoietic and CD33+ GM-Ps

Peripheral blood monocytes are clearly an important site of HCMV latency. However, these cells are short-lived, nonreplicating and are present in the circulation for only a brief period of time before entering the tissue and differentiating into MDM. These characteristics suggest another site of HCMV latency existing in a myeloid progenitor population that gives rise to latently infected CD14+ monocytes in the peripheral blood. HCMV has previously been reported to infect CD34+ pluripotent stem cells both in vitro (Goodrum *et al.*, 2002; Maciejewski *et al.*, 1992; Minton *et al.*, 1994; Movassagh *et al.*, 1996; Sindre *et al.*, 1996) and in vivo (Mendelson *et al.*, 1996; Torok-Storb *et al.*, 1992; von Laer *et al.*, 1995a,b). However, since CD34+ stem cells are a common precursor for all peripheral blood cell types, the absence of virus from many peripheral blood cell lineages suggests that CD34+ stem cells may not represent a major site for HCMV latency (Brytting *et al.*, 1995; Schrier *et al.*, 1995; Söderberg *et al.*, 1993; Taylor-Wiedeman *et al.*, 1993; von Laer *et al.*, 1995a,b). This observation would predict that a precursor population further differentiated along the myeloid pathway would be a more biologically significant site of HCMV latency in vivo. Alternatively, HCMV may alter CD34+ cell differentiation, leading to differentiation of only specific cell lineages, or the HCMV genome may be maintained only within specific cell lineages during differentiation.

CD33+ GM-Ps have been identified as one possible precursor population representing a more differentiated site of HCMV latency. Following in vitro infection, HCMV has been shown to infect and establish a latent infection in fetal liver-derived CD33+ GM-Ps (Kondo *et al.*, 1994; 1996; Hahn *et al.*, 1998). Virus was reactivated from CD33+ cells co-expressing either CD15 (granulocyte marker), CD14 (monocyte marker) or CD15 and CD1a (dendritic lineage marker) (Kondo *et al.*, 1994; Hahn *et al.*, 1998). IFN-γ and TNF-α reactivated HCMV from latently in vitro infected GM-Ps (Hahn *et al.*, 1998), closely linking reactivation with immune activation and cellular differentiation state as had been observed with Allo-MDM (Söderberg-Naucler *et al.*, 1997b). However, for both CD34+ precursors and CD33+ GM-Ps, conclusive evidence that these populations represent a site of HCMV latency in vivo awaits demonstration of HCMV reactivation from these cells in vitro following their natural infection in healthy individuals.

The mechanisms controlling HCMV latency and reactivation in these cell types are currently unknown. CMV latency-associated transcripts (CLTs) have been detected in latently infected GM-Ps suggesting a possible role of CLTs in the control of latency. CLTs are represented by sense (ORF94) and antisense transcripts (ORF152 and 154), expressed from the UL122/UL123 region of the genome. This region is normally involved in the expression of transcriptional activators (IE1 and IE2) that are required for lytic replication (Kondo *et al.*, 1996). The presence of CLTs in bone marrow aspirates from healthy HCMV seropositive individuals, as well as the presence of antibodies directed against proteins encoded by ORF94 and ORF152 in the serum of healthy HCMV seropositive individuals (Kondo *et al.*, 1996), have implicated CLTs in the control of HCMV latency in vivo. However, in the in vitro GM-P latency model, only a small proportion of latently infected GM-Ps have been shown to contain detectable CLTs (1.8%–3.6%) even though greater than 90% of GM-Ps are latently infected (Slobedman and Mocarski, 1999). Furthermore, inactivation of ORF94 does not affect the ability of latent HCMV to reactivate in this latency model (White *et al.*, 2000) and CLTs have never been observed in the Allo-MDM model. Together, these observations suggest that CLTs play a minimal role in the regulation of HCMV latency and reactivation. Recently, DNA array analysis using an *in vitro* CD34+ latency model has revealed an expression profile of HCMV genes that is unique to the latent, compared to lytic, state of infection (Goodrum *et al.*, 2002). The role of this latency-associated gene expression profile for the maintenance of latency in these cells is currently unknown. Further investigation may lead to the identification of HCMV ORFs involved in regulating virus reactivation.

Determinants of myeloid lineage cell tropism

Early studies identified differences in the ability of individual HCMV strains to infect monocytes and macrophages indicating that HCMV tropism for monocyte/MDM is genetically determined (Einhorn and Ost, 1984; Rice *et al.*, 1984). Although the genetic determinants for HCMV growth in MDM have not been identified, the MCMV model has provided considerable insight into the role of specific genes in CMV replication in MDM. In the murine model, initial

studies identified M45, M140 and M141 as virally encoded genetic determinants necessary for normal MCMV replication in macrophages in vitro (Brune *et al.*, 2001; Hanson *et al.*, 1999a,b 2001). In animal studies, M140 and M141 were shown to be essential for normal virus replication in the MDM-rich spleen, but not liver, indicating a role of these genes for determination of tropism in vivo (Hanson *et al.*, 2001). Importantly, viruses deleted of M140 and M141 had reduced pathogenicity indicating a role of MDM tropism in virus pathogenicity. Individual deletion of M140 and M141 resulted in decreased viral replication in macrophages; however, a low level of replication remained suggesting a redundancy of function or the existence of additional genes mediating virus replication in MDM. The M140 and M141 gene products are homologues of the HCMV US22 gene family. A study utilizing BAC-based technology has analyzed the function of the 12 US22 MCMV homologues in mediating macrophage tropism (Menard *et al.*, 2003). Results from this study confirmed the role of M140 and M141 as determinants of macrophage tropism, and identified two additional MCMV genes necessary for MCMV growth in macrophages (M36 and m139) (Menard *et al.*, 2003). However, M36 alone was required for growth in primary macrophages as well as macrophage cell lines. The identification of m139 as necessary for growth in macrophages contrasts with results from the earlier studies. This may be a consequence of sequence differences between viruses used by the different laboratories. The function of the HCMV US22 gene family members in mediating HCMV macrophage tropism has not been determined.

Role of apoptotic inhibitors in EC and MDM tropism

Similar to M45 (see above), the M36 product exhibits an anti-apoptotic function suggesting that an ability to inhibit apoptosis may be a common requirement for growth of cytomegalovirus in ECs and macrophages. Studies using a BAC-cloned recent clinical isolate (RVFIX) have shown that the HCMV M45 homologue (UL45) is not involved in the modulation of apoptosis and is dispensable for replication in HUVECs (Hahn *et al.*, 2002) (see above). However, the function of UL45 for replication in macrophages remains to be determined. Four additional HCMV genes, IE1, IE2, UL36/viral inhibitor of caspase-8-induced apoptosis (vICA) and UL37x1/viral mitochondria-localized inhibitor of apoptosis (vMIA) have also been shown to inhibit apoptosis following over-expression of recombinant protein (Goldmacher *et al.*, 1999; Skaletskaya *et al.*, 2001; Zhu *et al.*, 1995). All of these genes except UL36/vICA are essential for virus replication in fibroblasts (Goldmacher, 2002; Marchini *et al.*, 2001; Patterson, and Shenk, 1999) (observed at only low multiplicities of infection for IE1) (Greaves and Mocarski, 1998). The requirement of the HCMV M36 homologue (UL36/vICA) for replication in macrophages or ECs is unknown. However, the M36 and UL36/vICA products appear to share a similar function, with the products of both genes (pM36 and pUL36/vICA, respectively) inhibiting apoptosis at a comparable level within the apoptotic pathway (Menard *et al.*, 2003).

Results from a number of studies have given considerable insight into the mechanisms by which pUL36/vICA and the product of UL37×1/vMIA (pUL37×1/vMIA) inhibit the apoptotic pathway (Goldmacher, 2000; Skaletskaya *et al.*, 2001). The pUL36/vICA and pUL37×1/vMIA appear to function at distinct steps in the apoptotic pathway. During apoptosis, death stimuli (e.g., Fas-ligation) activate caspase 8 resulting in permeabilization of the mitochondrial membrane leading to the release of mitochondrial proteins into the cytoplasm. These mitochondrial proteins activate downstream caspases and nucleases leading to the apoptotic death of the cell. The pUL36/vICA appears to function early in the apoptotic pathway by binding to the prodomain of caspase 8 and inhibiting activation. In contrast, pUL37×1/vMIA is localized to mitochondria (Colberg-Poley *et al.*, 2000; Goldmacher *et al.*, 1999), and has been shown to prevent apoptosis by inhibition of BID-mediated permeabilization of the mitochondrial membrane (Goldmacher, 2002). The function of pUL36/vICA and pUL37×1/vMIA at separate steps in the apoptotic pathway is further supported by the ability of pUL36/vICA, but not pUL37×1/vMIA, to inhibit Fas-mediated apoptosis in type I cells where caspase 8 can directly activate caspase 3 and thereby bypass the mitochondria (Skaletskaya *et al.*, 2001). As indicated above, deletional analysis has shown that UL37×1/vMIA, but not UL36/vICA, is essential for HCMV replication in fibroblasts (Borst *et al.*, 1999; Goldmacher, 2002). Although UL37×1/vMIA is required for oriLyt-dependent viral DNA synthesis (Smith and Pari, 1995), the anti-apoptotic function of pUL37×1/vMIA appears to be critical for replication in fibroblasts, since pUL37×1/vMIA-deficient HCMV replication was rescued by co-expression of other apoptosis inhibitors (Goldmacher, 2002; Reboredo *et al.* 2004). Although the requirement of IE1, IE2, pUL36/vICA and pUL37×1/vMIA for HCMV replication has been determined in fibroblasts, the role of these proteins as determinants of HCMV tropism in other permissive cell types has not been investigated.

Summary

ECs and myeloid lineage cell populations are important sites of HCMV persistence and latency within the host.

The use of cells of the myeloid lineage as a virus reservoir closely links HCMV replication to the immune system, and the virus has exploited immune activation to provide the necessary signals for reactivation of latent virus. The utilization of in vitro culture systems is beginning to increase our understanding of reactivation, persistence and replication of HCMV within EC and myeloid cell populations. The ability of CMV to replicate in EC and MDM is determined by genetic determinants of the virus. The MCMV model has enabled identification of a number of genes that are critical for virus replication in these cells. Ongoing studies in a number of laboratories, in large part using BAC-cloned CMV, are further defining viral determinants of CMV tropism. Recently, a tropism island comprising UL128, Ul130, and UL131A has been identified as necessary for HCMV replication in ECs. These studies are expected to yield exciting insights into the necessary requirements for virus replication in these cells.

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Immunobiology and host response

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Introduction

HCMV, as all persistent viruses, has to survive in the host in the face of an immune response. Antibody, and probably T-cells in particular, contain the infection in the normal host but impaired T-cell immunity is associated with HCMV disease. The virus encodes functions which can counter this immune response and may also use immune cells as sites of latency. Although our knowledge of many aspects of the virus/host relationship is still incomplete, studies on HCMV over the past 20 years have given insight into how a large DNA virus achieves this coexistence with the normal immune response. Other chapters also contain relevant material.

Cells of the immune system as sites of latency and reactivation for HCMV

Consideration of the immune response to HCMV has to take account of the fact that some cells of the immune system are strong candidates for being sites of latency (see elsewhere in this volume). It is a longstanding clinical observation that HCMV can be transmitted by blood transfusion, but the most sensitive PCR based techniques do not detect HCMV DNA in plasma or serum of healthy virus carriers (although they do in patients with active HCMV disease), implying HCMV is most likely transmitted by cells in peripheral blood. Evidence from several laboratories suggests that HCMV is latent in myeloid lineage cells (Sinclair and Sissons, 2006). Using highly sensitive PCRs (of considerably greater sensitivity than those used for diagnostic assays) HCMV DNA can be detected in CD14+ peripheral blood mononuclear cells and in CD34+ cells in bone marrow (Mendelson *et al.*, 1996; Taylor-Wiedeman *et al.*, 1991). HCMV DNA is also reported in CD33+ cells in bone marrow (Hahn *et al.*, 1998; Kondo *et al.*, 1994). No infectious virus can normally be detected in freshly isolated peripheral blood monocytes or myeloid lineage progenitors, although the induction of HCMV immediate early gene expression can be detected by highly sensitive RT PCR when peripheral blood monocytes are differentiated with interferon-γ (IFN-γ) and GM CSF (Taylor-Wiedeman *et al.*, 1994). In order to demonstrate that a particular cell type is really harboring latent virus capable of reactivation, it is necessary to show that infectious virus can be rescued from such putatively latently infected cells. One group has reported that differentiation of peripheral blood monocytes from seropositive subjects to monocyte-derived macrophages in vitro in the presence of allogeneic T cells results in detection of infectious HCMV by co-cultivation of the cells with fibroblasts (Soderberg-Naucler *et al.*, 1997, 2001). There is also evidence that dendritic cells (DC) may be a specific site of latency and that their differentiation to mature DC in vitro may be associated with reactivation (Reeves *et al.*, 2005).

These observations all relate to the detection of endogenous HCMV DNA in cells of normal seropositive subjects, rather than the detection of HCMV which has been exogenously added to such cells during in vitro culture. Although the latter type of experiment with exogenous HCMV in general supports the evidence from detecting endogenous HCMV DNA that myeloid lineage cells are a site of persistence, it is always difficult to exclude the possibility that low level virus replication (rather than true latency) is maintaining HCMV DNA in such experimental in vitro systems.

Considering all of the current evidence from the literature, the most plausible picture of HCMV latency and reactivation in cells of the bone marrow and blood is that HCMV is carried in myeloid lineage progenitor cells in the bone marrow and is maintained in the cells as they divide down the myeloid lineage into peripheral blood monocytes and dendritic cells, albeit at a very low frequency – perhaps 0.01% of mononuclear cells – and

virus then reactivates when latently infected monocytes differentiate into macrophages (probably after exiting the circulation into tissues) under the influence of cytokines. However, unanswered questions include the following. (i) Is there a reservoir of HCMV in bone marrow which maintains virus in some stem cell population ? (ii) What is the mechanism by which viral DNA is maintained in myeloid lineage cells as they divide ? (iii) Is there a subpopulation of mononuclear cells, particularly DC, which is critical for maintenance of HCMV latency? (iv) What are the precise cytokines and pathways which differentiate peripheral blood mononuclear cells into the cell phenotype needed to permit reactivation of latent virus ? (v) Are there a limited set of HCMV RNA transcripts that are necessary for the maintenance of HCMV latent DNA (analogous to the LATs of HSV)?. There are reports of HCMV latency-associated transcripts in myeloid lineage cells in both natural and experimental infection, but it is unclear whether they are truly specific for latency and what their function might be (White *et al.*, 2000; Bego *et al.*, 2005). The subject is further discussed in the chapter by Nelson and colleagues.

The balance of evidence suggests that HCMV is not latent in other cell types in peripheral blood (Taylor-Wiedeman *et al.*, 1993). In healthy carriers, lymphocytes and peripheral blood neutrophils appear not to carry virus – although neutrophils may contain CMV antigens during active HCMV disease, there is no evidence they do this in normal seropositive subjects and the evidence is against their being a site of replication for the virus (Sinzger *et al.*, 1996).

The immune response to HCMV in the human host

Lessons from animal models of CMV disease

Murine cytomegalovirus (MCMV) infection of inbred mice has been extensively utilized as a model system to help to further understand HCMV infections, including aspects of immune responses, latency and immunopathology. This work has generated a substantial literature and made an important contribution to our understanding of the immunobiology of HCMV. The work cannot be reviewed here but is well summarized in several excellent recent reviews on the immune response to (Polic *et al.*, 1998; Reddehase, 2002), and latency of (Reddehase *et al.*, 2002), MCMV.

Antibody and complement

Following primary infection, antibodies specific to numerous HCMV encoded proteins are readily detectable in serum (for review see Britt, 1991; Landini and Michelson, 1988). These include structural tegument proteins (such as pp65 and pp150), envelope glycoproteins (predominantly gB and gH) as well as non-structural proteins such as the Immediate Early 1 protein (IE1, UL123) and a DNA binding protein (UL44). Viral neutralizing activity in vitro is predominantly mediated by antibodies specific for gB and gH (Britt *et al.*, 1988; Urban *et al.*, 1996). Both mouse and guinea pig animal models suggest that antibody is important in protection from a lethal infective dose and in reducing fetal infection (Rapp *et al.*, 1992; Harrison *et al.*, 1995). In humans, pre-existing humoral immunity to cytomegalovirus also plays an important role in preventing congenital infection of the fetus during pregnancy (Fowler *et al.*, 1992) and in preventing transfusion-associated infection in premature infants (Yeager *et al.*, 1981). The role of humoral immunity in the pathogenesis of HCMV disease in immunosuppressed patients is uncertain. Prior infection and thus the existence of preformed antibody to HCMV may be an important factor in limiting reactivation and protecting from re-exposure: however there is very limited evidence for the benefits of administration of HCMV-specific antibody to immunosuppressed transplant patients who are undergoing a primary infection or reactivation, with some studies reporting beneficial results and others suggesting no benefit (Guglielmo *et al.*, 1994; Munoz *et al.*, 2001).

gB in its mature form is composed of two disulfide bonded subunits gp116 and gp58 derived by the proteolytic cleavage of a glycosylated precursor molecule (Britt and Mach, 1996). The vast majority of HCMV-infected individuals mount an antibody response directed against gB, and a large proportion of neutralizing antibodies produced in response to viral infection are gB-specific (Britt *et al.*, 1990; Gonczol *et al.*, 1991; Marshall *et al.*, 1992; Schoppel *et al.*, 1997). The antibody response to gB has consequently been extensively studied, and three antigenic domains have been identified AD-1 (aa 552–635), AD-2 (aa50–77) and AD-3 (aa783–906). AD-2 has been further defined and is composed of two antibody binding sites, Site I (aa68–77) and Site II (aa50 to 54). Antibodies which bind AD-1 and AD-2 site II mediate viral neutralization (Kniess *et al.*, 1991; Meyer *et al.*, 1992; Wagner *et al.*, 1992). The AD-1 site is large, consisting of a run of 75 amino acids: within this run, cysteine residues at positions 573 and 610 form a disulfide bond which is required for binding of antibodies directed at AD-1 (Speckner *et al.*, 1999).

Of possible relevance to humoral immunity to HCMV is the existence of virus-associated Fc receptors. It has been recognized for some time that HCMV-infected cells are able to bind IgG isotype from human and other species, independent of any specific antibody activity to HCMV (Furukawa *et al.*, 1975; Keller *et al.*, 1976; Mackowiak and

Marling-Cason, 1987). However only recently has this IgG Fc binding activity been ascribed to viral encoded gene products rather than virus-induced cellular Fc binding proteins. Two proteins have been described to date. The first is a 34 KDa Fc binding protein (Fc-BP) encoded by the TRL11 and IRL11 ORFs and predicted to be a type I membrane glycoprotein with no homology to other herpesvirus encoded viral Fc binding proteins (Lilley *et al.*, 2000). The second protein is 68KDa and also a type I membrane glycoprotein encoded by the UL119-UL118 genes involving mRNA splicing (Atalay *et al.*, 2002). These may represent one of the many levels at which HCMV is modulating the host immune response – it has been suggested that binding of IgG could reduce the levels of neutralizing antibody directed at the virus. These Fc-BPs proteins have a rapid rate of endocytosis and endolysosomal degradation providing for an efficient antibody capture and disposal system. Binding of HCMV-specific IgG via the Fc region might also interfere with complement activation and antibody-dependent cellular cytotoxicity (Atalay *et al.*, 2002).

The HCMV specific CD8+ T-cell response

Given the inferential evidence for the importance of T cell immunity in controlling HCMV infection, and the evidence for the importance of the CD8+ T-cell response to MCMV, more attention has focussed on the CD8+ T-cell response to HCMV than on any other aspect of the immune response. HCMV specific CD8+ cytotoxic T-lymphocytes (CTL) were first detected in the peripheral blood lymphocytes (PBL) of patients with overt HCMV infection in the early 1980s and a short time later MHC restricted HCMV specific CTL precursors (CTLp) were stimulated to grow and mediate cytotoxicity from the PBL of normal healthy HCMV carriers (Borysiewicz *et al.*, 1983). Since then a large body of work has identified individual viral encoded proteins which are recognized by T-cells, defined minimal antigenic peptides within these proteins together with their MHC Class I restrictions, enumerated the frequency of HCMV-specific CD8+ T-cells in normal healthy carriers as well as immunosuppressed transplant patients, and analyzed in detail the clonal composition of HCMV specific CD8+ T-cells and the phenotypic characteristics of these long-term memory T-cells.

The cytomegalovirus gene products that are targets for the human CD8+ T cell response have been studied for over 20 years. Most recently a study produced overlapping peptides to the whole HCMV proteome (213 open reading frames) in order to comprehensively map all CD8 and CD4 epitopes. Previous to this work.

CD8+ T-cells specific for IE1 (UL123), the viral matrix proteins (pp65 (UL83), pp150 (UL32), pp28 (UL98), pp50 (UL44)), the surface viral glycoproteins (gB (UL55), gH (UL75)) and a number of other viral proteins (US2, US3, US6, US11, UL16 and UL18) have been detected using either interferon-γ secretion (ELISPOT or intracellular flow cytometry), or cytotoxicity using either bulk polyclonal T cell populations or T-cell clones as effector cells in radioactive Chromium release cytotoxicity assays (Borysiewicz *et al.*, 1983; Wills *et al.*, 1996; McLaughlin-Taylor *et al.*, 1994; Elkington *et al.*, 2003; Kern *et al.*, 2002; Longmate *et al.*, 2001).

The work of Riddell and colleagues first suggested a large proportion of the CD8+ T-cell response to HCMV was directed at the lower matrix protein pp65: these workers generated CD8+ T-cell clones for infusion into bone marrow transplant recipients by secondary in vitro restimulation of PBMC from their HCMV seropositive donors, and found the majority of clones were specific for this protein (Walter *et al.*, 1995). A number of subsequent studies concluded that the majority of the CD8+ T-cell response to HCMV in a given virus carrier is directed at pp65 (Wills *et al.*, 1996; Boppana and Britt, 1996). By comparing the killing of target cells infected with AD169 strain HCMV or a pp65 gene deletion mutant (RVAd65) in limiting dilution assays, the contribution of pp65-specific CTLp to the total HCMV-specific CTLp frequency was quantified in healthy seropositive virus carriers at between 70 and 90%, suggesting that pp65 is an immunodominant viral antigen. CD8+ T-cells specific for IE1 have also been isolated from seropositive carriers, although the frequencies of IE1-specific CTLp have been estimated as being on average ten fold lower than those for pp65-specific CTLp (Wills *et al.*, 1996). pp150-specific CTL have also been isolated from some donors at about a four fold lower frequency as compared to pp65. The frequency of CTLp specific for gB, gH, pp50 or pp28 has been reported as < 10 CTLp/million CD8+ T cells which is at the limit of detection of this assay (Boppana and Britt, 1996).

Estimations of the frequency of virus-specific CTLp in assays based on in vitro restimulation with antigen depend on the CTL precursors being exposed to all the appropriate CTL epitopes. When autologous HCMV-infected fibroblasts are used for restimulation in vitro, viral encoded proteins (US2, US3, US6 and US11) which interfere with antigen processing and presentation (Reddehase, 2002) may lead to selective presentation of particular virus peptides by surface MHC Class I, with a consequent underestimation of CTLp frequencies against certain antigens. The view that pp65 is immunodominant has been refined in the light of further data over the past few years. Panels of synthetic peptides spanning complete proteins combined with rapid epitope screening assays (based on intracellular

IFN-γ production detected by flow cytometry) have been used to reassess the frequency of IE1-specific CD8+ T-cells (Kern *et al.*, 2002). Previous observations that only a proportion of virus carriers have CD8+ T-cell responses to IE1 were confirmed, but this work also found much higher frequencies of IE1-specific T-cells similarly high to those specific for pp65 (Kern *et al.*, 2002). It has been shown that *de novo* protein synthesis is not required to sensitize target cells to killing by pp65-specific CTL (McLaughin-Taylor *et al.*, 1994): pp65 is present in virions as a preformed structural protein which is delivered to the cytosol during virus infection, from which pp65 can enter the MHC Class I antigen processing pathway. In contrast IE1 has to be synthesized *de novo* after virus infection at a time in the virus replication cycle when the ability of the infected cell to present new MHC Class I-peptide complexes is impaired. Direct stimulation of CD8+ T-cells using synthetic peptides in the absence of any influence of other viral proteins, has demonstrated much higher frequencies of IE1-specific CD8+ T-cells than previously reported and this may hold true for other HCMV antigens. A recent investigation utilizing a bioinformatics approach to predict CD8+ T-cell epitopes has described dozens of new HCMV T-cell epitopes within 14 HCMV encoded proteins. The new epitopes were verified using an ELISPOT assay measuring IFN-γ production, and some of the epitopes were also used to generate functional CTL (Elkington *et al.*, 2003). Although this approach clearly indicates that many other HCMV-encoded proteins can act as CD8+ T cell antigens, this work also confirms the previous findings that pp65-specific and IE-specific T-cells dominate the CD8+ T-cell response in most individuals.

In a recent and comprehensive study 13 687 consecutive 15-mer peptides overlapping each other by 10 amino acids and comprising 213 potential ORFs were synthesized and used in intracellular IFN gamma assays in order to identify all ORFs from HCMV that could be recognized by either CD4+ or CD8+ T cells derived from a cohort of 33 HCMV positive donors of diverse HLA haplotypes. The results showed that 47% of HCMV ORFs were recognized by CD8 T cells from at least one of the 33 donors, and on average, each donor exhibited CD8+ T cell responses to 7 different ORFs. Traditionally studied ORFs like pp65 (UL83) and IE1 (UL123) were among the most common, being recognized by more than half the subjects: however this study showed that many other ORFs are also commonly recognized and pp65 and IE1 are by no means universally immunodominant in such a diverse cohort (Sylwester *et al.*, 2005).

The production of soluble tetrameric MHC class I molecules loaded with specific viral peptide, then biotinylated and labeled with a fluorescent tag, provides a reagent which binds directly to the T-cell receptor (TCR) of peptide-specific CD8+ T-cells. These labelled Class I tetramers allow the visualization of antigen-specific T-cells by flow cytometry and have made an important contribution to the analysis of virus-specific T-cell responses to many human virus infections including HCMV. Tetramers have been constructed to pp65 peptides presented by HLA-A0201, A2402, B0702 B0801 and B3501 (Gillespie *et al.*, 2000; Hassan-Walker *et al*, 2001; Kuzushima *et al.*, 2001), and IE1 peptides presented by HLA-A0201 and B0801 (Khan *et al.*, 2002a,b; Wills *et al.*, 2002), and used to determine the frequencies of virus-specific CD8+ T-cell frequencies in a large number of HCMV seropositive subjects. The results confirm the very high frequency response to pp65 and IE1 in most HCMV seropositive subjects and also demonstrate that functional assays such as LDA tend to underestimate the HCMV-specific frequency (as has been noted in other virus sytems).

It is clear that primary HCMV infection elicits strong virus-specific CD8+ responses to numerous viral proteins: evidence suggesting that these specific CD8+ responses are protective is provided both by murine models of CMV, and by data obtained from patients undergoing reconstitution of their immune systems following bone marrow transplantation (BMT) or stem cell transplantation. Mice are protected from lethal MCMV challenge by CD8+ cells specific for immediate early antigens (Reddehase *et al.*, 1987). In a murine model of CMV reactivation (deficient in B-cell responses) it has been demonstrated that CD4+ and NK cells can substitute for CD8+ T-cells. However in murine models of BMT, removal of reconstituted CD8+ cells leads to lethal disease and reconstituted CD8+ cells transferred to immunocompromised mice could prevent disease (Polic *et al.*, 1998). Following BMT in humans there was a strong correlation between the recovery of cytolytic T-cell activity and recovery from HCMV infection. Subsequent studies quantifying T-cell recovery using virus-specific tetramers estimate that a recovery of virus-specific cells of >10 per μl of blood was protective against serious HCMV disease (Cwynarski *et al.*, 2001).

The availability of HCMV-specific MHC Class I tetramers, monoclonal antibodies against cell surface molecules and multi-parameter flow cytometry, and techniques for T-cell receptor analysis have made possible the detailed characterization of antigen-experienced HCMV-specific CD8+ T-cells.

Analysis of the clonal composition of the memory CD8+ T-cells specific for defined pp65 epitopes by sequencing of the TCRs of multiple independently derived epitope-specific CTL clones, reveals a high degree of clonal focusing: in a given virus carrier, the majority of CTL clones

specific to a defined pp65 peptide use only one or two different TCRs at the level of the nucleotide sequence. Thus in a given carrier the large population of circulating HCMV peptide-specific CD8+ T cells is in fact composed of only a few individual CD8+ clones that have undergone extensive clonal expansion in vivo (Weekes *et al.*, 1999). It is also clear that cells of an individual antigen-experienced pp65-specific clone persists in the virus carrier for years, because the same clones can be repeatedly isolated over time. CD8+ T-cells obtained from unrelated subjects that recognize the same defined peptide–MHC complex often use the same TCR Vβ segment, and have similar amino acid sequences within the hypervariable VDJ region of the TCR that binds to the viral peptide. Similar observations of clonal T cell focusing have also been made for CTL specific for a number of IE1 peptides (Khan *et al.*, 2002a,b). Other persistent virus infections (EBV and HIV) are also associated with this tendency to develop large oligoclonal T-cell populations. The focused pp65-specific and IE-specific CD8+ T-cell response observed in HCMV carriers may be the result of repeated exposure to viral antigen upon periodic reactivation of HCMV, with selection of CD8+ T-cells that express certain high affinity TCRs, and persistence of these clones in long-term memory. It is interesting to note that a study of HCMV-specific T-cell responses in elderly subjects showed that the frequency can increase over time to very large numbers (up to 25% of all CD8+ cells in an individual) and that the T-cell repertoires become more oligoclonal (Khan *et al.*, 2002a,b). It remains unclear how soon after primary HCMV infection the pattern of CD8+ T cell clonal focussing develops and to what extent the relative clonal dominance changes in long-term virus carriers. Following allogeneic BMT from HCMV-seropositive donors to HCMV-seropositive recipients, individual dominant HCMV-specific CD8+ clones present in the donor are typically transferred in the allograft, undergo expansion and are maintained long-term in the recipient; in addition, delayed emergence of different donor-derived clonotypes can be observed more than 6 months after transplantation (Gandhi *et al.*, 2003).

Detailed analysis of the phenotype of peptide-specific CD8+ T-cells has been undertaken using oligonucleotide clonotype probing (which can quantify a virus-specific T-cell receptor rearrangement directly from peripheral blood) or HCMV-specific tetramers, in combination with monoclonal antibodies to cell surface markers. The expression of the CD45RA and CD45RO isoforms of CD45 was previously thought to distinguish naïve (CD45RAhigh) from memory T-cells (CD45ROhigh). However in healthy carriers, cells of a single antigen-experienced HCMV-specific CD8+ T-cell clone are present in both the CD45RO(high) and CD45RA(high) T-cell subpopulations. During primary HCMV infection, all the highly activated effector HCMV-specific T-cells express CD45RO, but during convalescence they accumulate in the CD45RA(high) subpopulation consistent with reversion from CD45RO(high) cells (revertant memory) (Wills *et al.*, 1999). The same phenomenon has also been described following primary EBV infection (Faint *et al.*, 2001). Further analysis of the CD45RA(high) HCMV-specific T-cells using markers to cell surface adhesion, costimulation and chemokine receptor molecules has shown that they lack the costimulatory molecule CD28 and the chemokine receptor CCR7. However, in contrast to previous suggested models of CD8+ T-cell memory, these CD28(−) CD45RA(high) CCR7(−) cells are not terminally differentiated, because following stimulation in vitro with specific HCMV peptide these cells undergo sustained clonal proliferation, up-regulate CD45RO and CCR5, and show strong peptide-specific cytotoxic activity. In an individual with acute primary HCMV infection, HCMV pp65-specific CD8+ T-cells are predominantly CD28(−) CD45RO(high) CCR7(−). During convalescence, an increasing proportion of pp65-specific CD8+ T-cells were CD28(−) CD45RA(high) CCR7(−). Thus CD8+ T-cell memory to HCMV is maintained by cells of expanded HCMV-specific clones that show heterogeneity of activation state and costimulation molecule expression within both CD45RO(high) and CD28(−)CD45RA(high) T-cell pool (Wills *et al.*, 2002). Similar techniques have been used by a number of groups to examine the memory T-cells generated in response to EBV: it is interesting to note that T-cells specific for lytic EBV antigens display very similar phenotypic profiles to HCMV pp65-specific T-cells. It is also of interest that this does not hold for all persistent virus infections (Faint *et al.*, 2001; Appay *et al.*, 2002).

The capacity of viruses to encode functions which may enable them to evade the effector function of cytotoxic T-cells has generated enormous interest over the last 5 years. Investigation of the ways in which HCMV can disrupt the normal MHC Class I antigen processing pathways has revealed a surprising number of mechanisms: these include the degradation of newly synthesised MHC Class I heavy chains (mediated by US2 (Wiertz *et al.*, 1996b) and US11 (Wiertz *et al.*, 1996a; Jones *et al.*, 1995), retention of MHC Class I peptide complexes in the endoplasmic reticulum (ER) (mediated by US3 (Jones *et al.*, 1996) and blockade of peptide translocation into the ER (mediated by US6 (Ahn *et al.*, 1997). Further details are available elsewhere in this volume and in a recent review by Reddehase *et al.* (2002). The sequential expression of the US3 and US11 gene products which would lead to MHC Class I-peptide complex retention in the ER (US3)

followed by degradation of de novo MHC Class I heavy chains (US11) may be very efficient. The combination of US2 and US11 may allow many different MHC Class I heavy chains (as would be found in an outbred population) to be redirected to the cytosol for degradation. HCMV infects and becomes latent in a number of different cell types in vivo and it is conceivable that some of these viral gene products are more efficient in some cell types as compared to others. The human immune system and HCMV have co-evolved to a considerable extent, and it is possible that the diversity of immune evasion mechanisms reflects this co-evolution of the virus and the immune system, and that some of the immune evasion mechanisms are vestigial.

It is clear that in spite of viral immune evasion genes, upon primary HCMV infection the host mounts a strong T-cell response composed of both CD8+ CTL and CD4+ helper T-cells which produce antiviral cytokines: the outcome is that acute primary infection is resolved, although HCMV is not cleared from the host but becomes latent with periodic reactivation and production of new virions. It is also clear that during long term carriage of the virus there is a balance between the cell-mediated immune response and viral activity. This balance is lost in the immunocompromised host where reactivation of latent virus or primary infection can lead to uncontrolled viral replication which may cause serious disease and death. Although the functional significance of the immune evasion genes in vivo remains to be determined, a plausible hypothesis is that the immune evasion mechanisms give the virus a "window of protection" during reactivation from latency in the face of an expanded population of antigen-experienced T-cells, enabling the virus to complete its life cycle to produce new virions. During reactivation the IE proteins are abundant at a time when the virus is retaining MHC Class I complexes in the ER, blocking antigen presentation and preventing T-cell surveillance. The virus may thus be able to replicate and release progeny virus from the cell: however, subsequent infection of neighboring cells will deliver preformed viral structural proteins to the cytosol which can enter the antigen processing pathway and be presented at the infected cell surface for T-cell recognition, resulting in local containment of the reactivation episode.

The HCMV-specific CD4+ T-cell response

CD4+ T-cells play a key role in the control of virus infections, by the activation of dendritic cells (by ligation of CD40 on the dendritic cell by CD154 on the T-cell), by providing help for virus-specific B-cells (ligation of CD40 on the B-cell induces immunoglobulin gene somatic hypermutation and class switching), and by secreting cytokines that facilitate the proliferation and differentiation of virus-specific CD8+ T-cells. In murine CMV infection, long-term selective depletion of CD4+ T-cells in vivo is associated with persistent virus replication at specific sites (Jonjic *et al.*, 1989). Analysis of the HCMV-specific CD4+ T-cell response in humans has been greatly enhanced by the development of flow cytometry methods to detect intracellular cytokine expression by antigen-specific T cells (Waldrop *et al.*, 1997, 1998). At present the production of peptide-MHC Class II tetramers to identify HCMV-specific CD4+ T-cells is technically demanding; when such tetramers become widely available, not only the analysis but also the therapeutic manipulation of HCMV-specific CD4+ T-cells may become possible in the future.

Using in vitro restimulation of PBMC with whole HCMV antigen and detection of intracellular cytokine expression by flow cytometry, in healthy HCMV carriers typically 1%–2% of all circulating CD4+ T-cells are specific for HCMV (Waldrop *et al.*, 1998; Rentenaar *et al.*, 2000) with one report of much higher frequencies (Sester *et al.*, 2002). In approximately 60% of HCMV carriers, virus-specific CD4+ T-cells recognize the lower matrix protein pp65, and in a given carrier, CD4+ T-cells typically focus on one or a few peptide epitopes within pp65 (Beninga *et al.*, 1995; Kern *et al.*, 2002). The recent examination of the whole HCMV proteome for CD8 T cell specific responses also included CD4+ T cell responses: the results show that 44 unique ORFs are antigens for CD4+ T cells and that 81 ORFs are targets for both CD4 and CD8 responses (Sylwester *et al.*, 2005). In part because fewer peptide binding motifs are known for MHC Class II alleles than for MHC Class I alleles, relatively few MHC Class II restricted epitopes within pp65 have been identified (Kern *et al.*, 2002; Khattab *et al.*, 1997; Le Pira *et al.*, 2004; Weekes *et al.*, 2004).

In healthy virus carriers, the surface phenotype of HCMV-specific CD4+ T-cells has been analyzed by staining of HCMV-stimulated PBMC with two or three different fluorochrome-linked monoclonal antibodies. CD4+ T-cells responding to stimulation by whole HCMV are enriched within CD45RO+, CD27−, CD62L−, CD11a^{high} and CCR7− subpopulations (Rentenaar *et al.*, 2000; Sester *et al.*, 2002; Bitmansour *et al.*, 2002). Most peripheral blood HCMV-specific CD4+ T-cells secrete IFN-γ, a proportion of which also secrete TNFα and IL-2; very few HCMV-specific CD4+ T-cells secrete IL-4 (Rentenaar *et al.*, 2000; Bitmansour *et al.*, 2002). Many HCMV specific CD4+ cells are also cytotoxic and MHC Class II restricted (van Leeuwen *et al.*, 2006; Weekes *et al.*, 2004).

In healthy HCMV carriers, the circulating population of HCMV-specific CD4+ T-cells is oligoclonal. Following in

vitro stimulation with whole HCMV antigen, responding HCMV-specific CD4+ T-cells showed striking focussing of TCR Vβ segment usage by monoclonal antibody staining. When HCMV-specific CD4+ T-cells were sorted and analyzed by RT-PCR, the TCR Vβ expansions were composed of a limited number of clonotypes in which 1–3 clones dominated the response together with a cohort of subdominant clones and numerous minor clones. The same dominant clonotypes were identified when CD4+ T-cells were stimulated with individual pp65 peptides (Bitmansour *et al.*, 2002). Cells of an individual expanded clone showed a spectrum of triggering thresholds consistent with TCR-independent threshold regulation, including different thresholds for secretion of IFN-γ and IL-2, but the triggering thesholds did not differ between CD27+ and CD27− cells of the given clonotype. These results indicate that a given antigen-experienced CD4+ clonotype can give rise to qualitatively distinct functional responses depending upon epitope dose and availability of costimulation (Bitmansour *et al.*, 2002).

To study the CD4+ T-cell response during natural primary HCMV infection in immunocompetent subjects is difficult; usually the time at which infection began is uncertain, and subjects have often had symptoms for a number of days before primary HCMV infection is confirmed by detection of anti-HCMV IgM. Longitudinal studies of primary HCMV infection have been performed in the setting of HCMV-seronegative individuals who received a renal transplant from a HCMV-seropositive donor (D+/R−) without prophylactic Ganciclovir treatment, although the introduction of HCMV via a solid organ that lacks lymphatic drainage and the concurrent use of immunosuppressant therapy (to prevent graft rejection) may modify the kinetics of infection and of the host T cell response (Rentenaar *et al.*, 2000; Gamadia *et al.*, 2003). HCMV DNAemia was first detected at a median of 25 days (range 18–29 days) after transplantation, and HCMV-specific IFN-γ-producing CD4+ T-cells were first detected (by intracellular cytokine staining following in vitro restimulation with whole HCMV antigen) at a median of 7 days (range 4–14 days) after first detection of HCMV DNAemia. In four of five subjects, HCMV-specific CD4+ T-cells were detected 3–10 days before anti-HCMV IgM. The HCMV-specific CD4+ T cell response developed rapidly, reached peak frequencies of 0.46% to 2.5% of peripheral blood CD4+ T-cells, and decreased rapidly to a low level over at least the next 10 weeks. In contrast to the phenotype observed in long-term virus carriers, during primary infection HCMV-specific CD4+ T-cells were predominantly CD38+, many were CD27+, and most showed co-expression of CD45RO and CD45RA. During primary infection a proportion of HCMV-specific CD4+ T cells were in cell cycle, as indicated by Ki67 expression (Rentenaar *et al.*, 2000). In a second study, compared to asymptomatic primary infection, symptomatic primary HCMV infection was associated with delayed appearance of HCMV-specific CD4+ T-cells in peripheral blood. Among nine subjects who developed primary HCMV infection following D+/R– renal transplantation, four subjects had symptomatic infection that required Ganciclovir treatment, while the other five subjects had asymptomatic infection. In the symptomatic subjects the time at which HCMV DNAemia was first detected after transplantation (median 27 days) was similar to that in asymptomatic subjects, but the peak viral load and duration of HCMV DNAemia was greater in the symptomatic subjects. The time from first detection of HCMV DNAemia to first detection of HCMV-specific CD4+ T-cells by intracellular cytokine staining was significantly longer in symptomatic infection (median 39 days, range 28–53) compared to asymptomatic infection (median 10 days, range 0–17). In symptomatic subjects, HCMV-specific CD4+ T-cells only became detectable after starting Ganciclovir therapy, and reached peak frequencies of 0.36% to 1.42% of peripheral blood CD4+ T-cells. There were no differences in the kinetics of antibody or CD8+ T-cell responses between symptomatic and asymptomatic subjects; the time from first detection of HCMV DNAemia to first detection of either anti-HCMV antibody (15 days vs. 17 days) or HCMV-specific CD8+ T-cells by peptide MHC Class I tetramer staining (21 days vs. 24 days) was similar in symptomatic and asymptomatic subjects (Gamadia *et al.*, 2003).

HCMV, innate immunity and natural killer (NK) cells

NK cells were originally described for their ability to mediate cytotoxicity against certain tumors without prior activation; they are characterized by the lack of both T- and B-cell markers, and are a component of the innate immune system. An important role for NK cells in the early control of viral infections is emerging (Tay *et al.*, 1998; Biron *et al.*, 1999). There is only limited evidence for the role of NK cells in the control of HCMV infection (Biron *et al.*, 1989, 1999). However the virus encodes a variety of NK cell evasion mechanisms which are discussed in this section, which provides indirect evidence for the importance of these cells in the innate response to HCMV. In the MCMV murine model system the evidence for their protective role is much stronger. Newborn mice are highly susceptible to lethal MCMV infection until NK responses become apparent at 3 weeks. Adoptive transfer of NK cells into these mice or adult SCID mice can confer protection reviewed in (Tay *et al.*, 1998). Inbred mouse strains have differing

resistance to MCMV (Scalzo *et al.*, 1992), and the dominant Cmv1 resistance locus has been mapped to the NK cell gene complex on chromosome 6. The Cmv1 resistance gene has now been shown to encode an activating NK cell receptor Ly49H (Brown *et al.*, 2001; Lee *et al.*, 2001; Daniels *et al.*, 2001).

Activation of NK cells is promoted by signals from activating NK receptors, but inhibited by signals received through the inhibitory NK receptors which interact with specific MHC class I molecules on the surface of the target cell. Individual NK cell clones express distinct patterns of inhibitory NK receptors, which include immunoglobulin supergene family members and C-type lectin family members (for review see Lanier, 2005). The C-type lectin heterodimer CD94/NKG2A is present on the surface of the NK cell and ligates HLA-E on a normal cell surface (Braud *et al.*, 1998). HLA-E is stabilized and translocated to the cell surface when it binds certain signal sequence peptides derived from the normal turnover of HLA-A, HLA-B and HLA-C molecules (Braud *et al.*, 1997).

As discussed earlier, HCMV encodes a number of genes responsible for the retention and destruction of MHC Class I molecules. Upon HCMV reactivation (Sissons *et al.*, 2002), the early interference with the MHC Class I pathway may impede the presentation of viral derived peptides to the host CD8+ T cells and hence avoid recognition. However, the reduced levels of surface Class I MHC on HCMV-infected cells might be expected to favor activation of host NK cells (Karre *et al.*, 1986). This has led to the suggestion that HCMV would also have to evade NK cell recognition in order to safeguard this window in which a latent genome within a cell can reactivate, and have enough time to assemble and release infectious virus. The observation in a number of experimental systems that HCMV-infected cells are relatively resistant to NK-mediated lysis suggests that HCMV has evolved viral encoded functions to evade NK surveillance (Reyburn *et al.*, 1997; Vales-Gomez *et al.*, 2003; Wang *et al.*, 2002; Cerboni *et al.*, 2000; Fletcher *et al.*, 1998; Arnon *et al.*, 2005; Tomasec *et al.*, 2005; Wills *et al.*, 2005). Six distinct virus encoded proteins and mechanisms have been proposed to prevent NK activation and lysis of HCMV-infected cells.

HCMV expresses a viral MHC Class I homologue gpUL18 which was reported to inhibit NK lysis (Reyburn *et al.*, 1997), although this report has been contradicted by a subsequent investigation which suggested that UL18 may activate NK cells (Leong *et al.*, 1998). These data might now be explicable in the light of the recent identification in MCMV of a new MHC Class I-like molecule (m157) which can engage an inhibitory NK receptor in one strain of mouse and an activating receptor in others (Arase *et al.*, 2002). It has subsequently been shown that HCMV UL18 binds LIR-1/ILT2 which is present on only a subset of NK cells (Cosman *et al.*, 1997). At present it remains unclear whether UL18 expression on HCMV-infected cells activates or inhibits specific populations of NK cells.

The viral protein encoded by HCMV UL40 also includes a signal sequence peptide similar to those of Class I molecules, and expression of UL40 has been reported to stabilize HLA-E (Tomasec *et al.*, 2000). HLA-E expression is not affected by the US2-11 gene products, and the maintenance of surface HLA-E expression by providing an appropriate signal peptide from UL40 has been proposed as another novel NK cell evasion strategy for HCMV (Wang *et al.*, 2002). However other groups have not observed this effect of UL40 in their systems (Falk *et al.*, 2002).

Some activating NK receptors recognize host cell proteins which are upregulated on the surface of stressed cells, for example, the activating receptor NKG2D recognizes MICA and MICB. A third mechanism of NK cell evasion is mediated by the viral membrane glycoprotein UL16 which is able to bind to MICB, ULBP-1 and ULBP-2 and sequester them in the endoplasmic reticulum/cis-Golgi, thereby preventing them interacting with NK cells bearing NKG2D and delivering an activating signal to the NK cell (Dunn *et al.*, 2003; Cosman *et al.*, 2001; Sutherland *et al.*, 2001).

A structural protein (pp65) from the virus tegument has been shown to interact with an activating NK receptor NKp30 leading to dissociation of the linked CD3zeta from NKp30 and thus reducing NK cell mediated killing (Arnon *et al.*, 2005).

In addition to these data, a comparison of the ability of different stains of HCMV to resist NK mediated lysis of infected fibroblasts has demonstrated a striking difference between the laboratory adapted strain AD169, and strains of the virus (including Toledo) that are more closely related to clinical isolates (Cerboni *et al.*, 2000; Fletcher *et al.*, 1998). As both laboratory adapted and clinical strains of the virus possess the UL16, UL18 and UL40 open reading frames these observations imply that other viral genes are responsible for this phenotype. Fletcher *et al.* (1998) reported that clinical strains of the virus downregulated lymphocyte function-associated antigen-3 (LFA-3) whereas AD169 did not, and speculated that this may be responsible for the difference in phenotype: however, Cerboni *et al.* (2000), while also observing the difference between clinical and laboratory strains, were not able to show any correlation with LFA-3 downregulation. It has been recognized since 1996 that clinical isolates of the virus have a larger genome, and comparison of AD169 with Toledo virus shows that Toledo has a 13.5 kb insert which encodes 20 predicted ORFs (Davison *et al.*, 2003) that are absent from AD169. It seems likely that

some of the genes encoded in the UL b' region are responsible for rendering infected fibroblasts resistant to NK lysis Two genes encoded in the Ulb' region have now been shown to mediate inhibition of NK cell lysis. ORF UL141 is able to inhibit NK cell mediated lysis in a clonally dependent manner, by sequestering CD155 in the ER and thus preventing it engaging an NK activating receptor DNAM-1 (Tomasec *et al.*, 2005). ORF UL142 is a novel HCMV encoded MHC class I related molecule which also inhibits NK cell killing in a clonally dependent manner. UL142 is also localized to the ER and it is likely that it sequesters virus induced stress molecules that would otherwise traffic to the cell surface to be recognized by activating NK cell receptors (Wills *et al.*, 2005).

The immune response and pathology in immunocompromised subjects

Immunosuppression by CMV

It is frequently stated that HCMV is "immunosuppressive." At a clinical level, the association is somewhat anecdotal: obviously CMV disease frequently arises in the context of immunosuppression, iatrogenic or otherwise, and it is difficult conclusively to implicate HCMV in the causation of immunosuppression. The disease associated with primary HCMV infection in the normal immunocompetent host is characterized by marked T cell proliferation, but not obviously associated with immunosuppression.

The possession of a large number of gene functions which modulate the expression of MHC molecules, cytokines and NK cell interactions, is really the most specific evidence that HCMV exerts "immunosuppressive" effects, although it is obvious these effects do not prevent the normal host from mounting a sustained and effective immune response against the virus. In the whole organism the effect of HCMV- induced downregulation of MHC molecules may be counteracted by other influences – for instance, it has been shown that IFN-γ and TNFγ can upregulate MHC Class I molecules and counteract the downregulating effect of the HCMV genes referred to above (Hengel *et al.*, 1995).

Much of the in vitro evidence for "immunosuppression" comes from experiments in which investigators have added HCMV to cultures of peripheral blood mononuclear cells and then used a readout such as T cell proliferation in response to exogenous mitogens or a similar assay. Given that HCMV, particularly recent clinical isolates, can infect differentiated macrophages, it can be envisaged that infection in this sort of in vitro system at high multiplicities may well cause impairment of such assays. More recently, it has been shown that HCMV can exogenously infect dendritic cells (DC) in vitro (Raftery *et al.*, 2001), with enhanced expression of their costimulatory molecules and partial downregulation of MHC molecules, with upregulation of apoptosis-inducing ligands CD95L (FasL) and tumor necrosis factor related apoptosis-inducing ligand (TRAIL). This would result in HCMV-infected DC potentially being able to delete activated T-cells. There is emerging evidence that dendritic cells may well be a site of HCMV latency and, although it can be envisaged this sort of mechanism might operate in vivo, it is difficult to know how valid it is to extrapolate from in vitro experiments in which large numbers of DC are infected to the situation likely to obtain in vivo, where HCMV infection of DC would seem likely to be a low frequency event. Murine DC have also been shown to be permissive for murine CMV infection in vitro: again, MCMV infected DC were unable to deliver the signals necessary for T-cell activation and it has consequently been suggested they may be involved in CMV-induced immunosuppression in the mouse (Andrews *et al.*, 2001).

In summary, the issue of whether HCMV can exert generalized immunosuppressive effects in vivo is unresolved. The clinical settings in which disseminated HCMV disease occurs are usually characterized by multiple variables operating simultaneously – such as pre-existing immunosuppression due to other disease or the administration of immunosuppressive drugs, the simultaneous administration of anti-rejection therapy, and other opportunistic infections. Given the in vitro evidence, it is plausible to suggest that CMV disease may be causally associated with immunosuppression but the case has to be regarded as not proven.

Immunopathology in human CMV disease

CMV pneumonitis

Pneumonitis is the most serious manifestation of HCMV infection after bone marrow transplantation (BMT), occurring in 10–15% of allogeneic BMT recipients, with a mortality of 80% prior to antiviral therapy. There is interstitial pneumonitis in the absence of any other identifiable pathogen, with increasing arterial hypoxemia, and progression to respiratory failure. It is suggested that graft versus host disease (GVHD) may contribute to the lung injury in HCMV pneumonitis in BMT recipients. The relationship between HCMV and GVHD is controversial, with proposals both that HCMV may predispose to GVHD, and vice versa. The striking rarity of pneumonitis attributable to HCMV in patients with AIDS, implies that factors other than immunosuppression alone contribute to its occurrence in BMT recipients. It has been hypothesized that CMV pneumonitis is mediated by an immunopathological mechanism, consequent on the regenerating immune system following bone marrow transplantation reacting against CMV infected cells in the lung. It has been suggested that CD4+ T

cells might be particularly involved and that the absence of CD4+ cells in HIV infection might explain the rarity of CMV pneumonitis in that setting: however, in fact cells recovered from bronchoalveolar lavage during CMV pneumonitis in bone marrow transplant patients are mainly NK cells and CD8+ cells. An alternative suggestion has been that uncontrolled virus replication might trigger a dramatic release of cytokines such as TNFγ and this might mediate the pneumonitis – although any direct evidence for this is lacking (Barry *et al.*, 2000).

Reddehase and colleagues (Podlech *et al.*, 2000) have used the model of syngeneic bone marrow transplantation and simultaneous infection of BALB/c mice with MCMV to study the mechanisms of CMV interstitial pneumonitis in a longitudinal fashion. These authors have taken care to adapt the mouse model to mimic as closely as possible the events surrounding allogeneic bone marrow transplantation in humans. When reconstituting CD8+T-cells were depleted, there was a disseminated cytopathic MCMV infection of the lungs with high mortality. When hematopoietic reconstitution with both CD8+ and CD4+ T-cells occurred, viral replication in the lungs was much less and restricted to focal areas – after clearance of the infection, memory CD8+ T-cells persisted in the lung tissue with little MCMV present. These authors concluded there is no evidence for CD8+ T-cells exerting an immunopathological effect, but rather of their protecting against MCMV infection in the lungs. They make the point that the late phase appearances with persisting memory cells in the lung could be misinterpreted as CMV-induced immunopathology and conclude that this mouse model provides no evidence for immunopathologically mediated interstitial pneumonitis in human BMT recipients.

CMV retinitis and immune recovery vitritis

HCMV disease is one of the most frequent opportunistic infections in patients with advanced HIV infection, of whom 40% develop sight or life threatening HCMV disease. A CD4+ T-cell count of $< 50/\mu l$ carries a particular risk of disease, although the widespread use of highly active antiretroviral therapy (HAART) in developed countries means that relatively few patients now have such low CD4+ T-cell counts, and the incidence of HCMV disease in patients with AIDS has consequently declined significantly. The commonest manifestation is HCMV retinitis which was seen in up to 25% of patients with AIDS prior to effective antiretroviral therapy. It is characterized by hemorrhagic retinal necrosis, spreading along retinal vessels, and threatening sight when disease encroaches on the macula (Whitcup, 2000).

However a newly recognized syndrome consequent on the use of HAART is "immune recovery vitritis." This is characterized by posterior segment inflammation and occurs in patients with inactive previously treated CMV retinitis, as the CD4+ T-cell count reconstitutes on antiretroviral therapy. In one series 60% of patients with prior HCMV retinitis who responded to HAART developed the syndrome (Karavellas *et al.*, 1999). Although attributed to infiltrating T-cells reacting to HCMV antigens in the eye, this mechanism (whilst plausible) is not yet proven, although T cells are present in histopathological specimens of epiretinal membrane in the disease. The inflammation responds to steroids alone.

CMV and inflammatory demyelinating neuropathy

The pathogen most frequently associated with acute inflammatory demyelinating neuropathy, also known as Guillain–Barré syndrome (GBS), is *Campylobacter jejuni*. However a proportion (5%–10%) of patients with GBS show serological evidence of primary HCMV infection, and are more likely to have IgM antibodies to the GM2 ganglioside than other patients with GBS (Khalili-Shirazi *et al.*, 1999): a causal relationship is postulated. CMV-infected fibroblasts have been shown to express the GM2 epitope (Ang *et al.*, 2000) and "molecular mimicry" has been postulated as a possible cause of the association (Yuki, 2001).

CMV and organ transplant rejection

The risk of HCMV disease is 3–5 times greater in a seronegative than a seropositive recipient receiving an organ allograft from a seropositive donor, and disease is much more severe. Many centers "match" seronegative donors to seronegative recipients, although this is often thwarted by organ shortage. Disease often presents with specific organ involvement not seen in the normal subject. Interstitial pneumonitis due to HCMV carries a poor prognosis; disease in the gastrointestinal tract includes oesophagitis, gastritis and gastric ulceration, and colitis; HCMV retinitis may occur in severely immunosuppressed patients (Rubin, 2001; van der Bij and Speich, 2001).

There has been much circumstantial suggestion in the literature that CMV, even more so than other virus infections, may be somehow involved in the pathogenesis of rejection of solid organ allografts. There is suggestive epidemiological evidence that clinically significant HCMV infection is commoner in subjects who develop graft rejection, although it can be difficult to dissect out whether HCMV infection is a consequence of the immunosuppressive therapy in use, or preceded the treatment of rejection episodes – the latter would make a causal association more plausible (Kashyap *et al.*, 1999; Borchers *et al.*, 1999). Possible mechanisms which have been invoked to explain how CMV might

be causally associated with rejection have focussed mainly on changes which the virus might induce in endothelial cells and in the expression of Class II MHC molecules. It is postulated that CMV-induced upregulation of adhesion molecules on endothelial cells might promote the infiltration of allospecific T-cells (Borchers *et al.*, 1999; Craigen and Grundy, 1996). There is some evidence for increased expression of Class II MHC molecules on cells in solid organs in association with CMV infection – given the evidence that, if anything, CMV may downregulate class II MHC molecules in isolated cell types in vitro, such upregulation seems more likely to be mediated by cytokine release related to virus infection, such as IFN-γ or others. There is some evidence from the model of rat CMV infection that rat CMV can enhance chronic kidney allograft rejection in a transplant model (Lautenschlager *et al.*, 1997) and that this is associated with increased vascular endothelial and tubular epithelial expression of ICAM-1 and increased interstitial inflammation (Yilmaz *et al.*, 1996).

One group has reported that CD13 (Aminopeptidase N, a cell surface zinc metalloproteinase) is incorporated into virions, and that this may be associated with the development of autoantibodies to CD13. In the allogeneic bone marrow transplant setting, the presence of antibodies to CD13 correlated with the development of GVHD, and it was suggested CMV induced CD13 specific autoimmunity was contributing to the mediation of the GVHD (Moller *et al.*, 1999).

The other aspect of solid organ allograft rejection in which CMV has been implicated is the chronic vasculopathy which may be a feature of chronic rejection. In cardiac transplantation, the most common cause of death following transplantation is cardiac allograft vasculopathy, an obliterative progressive vascular disease of the coronary arteries which is believed to be a form of chronic rejection (Orbaek Anderson, 1999). A number of studies have indicated an association between CMV and cardiac allograft vasculopathy (Weill, 2001), and it has been postulated that CMV may promote vasculopathy by the sort of mechanisms discussed above. Again, these suggestions are largely based on circumstantial evidence, although the virus may enhance the development of allograft vasculopathy in the rat CMV model of heterotopic heart or aortic transplantation, (Hosenpud, 1999; Koskinen *et al.*, 1999).

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Persistence in the population: epidemiology and transmisson

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Introduction

Cytomegaloviruses (CMVs) are ubiquitous but highly species specific agents and are a common cause of infections in many animal species including humans (Weller, 1971). The characteristic cellular changes caused by CMV including cell enlargement with intranuclear inclusions were first reported in 1881 by Ribbert in the kidneys of a stillborn infant with congenital syphilis (Ribbert, 1904). Subsequent reports have described similar findings in the parotid glands of children and in the salivary glands from guinea pigs. It was initially thought that cytomegalic inclusion disease (CID) of the newborn was the sole manifestation of human CMV (HCMV) infection (Goodpasture and Talbot, 1921; Lipschutz, 1921; Cole and Kuttner, 1926; Lowenstein, 1907). Several groups of investigators have simultaneously isolated and propagated HCMV from infants and children with CID and from adenoidal tissue of children undergoing adenoidectomy (Rowe *et al.*, 1956; Smith, 1956; Weller *et al.*, 1957). As tissue culture isolation and serological assays became more widely available, HCMV was linked to a variety of illnesses, many of which have subsequently been shown to be unrelated to HCMV. A common characteristic of patients at risk for invasive HCMV infections is the suppression of host immune responsiveness. The onset of AIDS epidemic in the early 1980s has led to a dramatic expansion of the spectrum of HCMV disease. HCMV was the most common opportunistic infection in patients with AIDS and a major cause of morbidity and mortality in these patients until the introduction of highly active antiretroviral therapy (Jacobson *et al.*, 1988; Gallant *et al.*, 1992; Munoz *et al.*, 1993; Spector *et al.,* 1999). Currently, HCMV continues to cause disease in patients with AIDS, but similar to other opportunistic infections in AIDS patients responding to antiretroviral therapy, the incidence of invasive HCMV disease is extremely low even in those with minimally reconstituted immune systems (Jacobson *et al.*, 2001). Currently, HCMV is a cause of significant morbidity and mortality in newborn infants who acquire HCMV prenatally and in allograft recipients.

Epidemiology of HCMV infection

Human cytomegalovirus infections have been recognized in every human population that has been studied (Krech *et al.*, 1971; Gold and Nankervis, 1976). HCMV infection is endemic without seasonal variation (Gold and Nankervis, 1976). HCMV is acquired early in life in most populations with the exception of people in economically well developed countries of northern Europe and North America. The patterns of HCMV acquisition vary greatly based on geographic and socioeconomic backgrounds of the population and the seroprevalence increases generally with age (Alford *et al.*, 1981). In the developing world, acquisition of HCMV is nearly universal in early childhood. Studies have shown that most preschool children (>90%) in South America, Sub-Saharan Africa, East Asia, and India are HCMV antibody positive (Gold and Nankervis, 1982; Stagno, 2001). In contrast, seroepidemiologic surveys in Great Britain and in certain populations in the United States have found that less than 20% of children of similar age are seropositive (Huang *et al.*, 1980; Gold and Nankervis, 1982).

In Chengdu, China a population survey observed 60% of children 4 to 7 years of age were HCMV seropositive (Liu *et al.*, 1990). Similiarly, 58% of children 4 to 12 years of age in Taipei, Taiwan, 61% of hospitalized pediatric patients from a low income population in Rio de Janeiro, Brazil and 56% of children aged 1 to 4 years in Jamaica were HCMV antibody positive (Prabhakar *et al.*, 1992; Shen *et al.*, 1992; Suassuna *et al.*, 1995). In Finland the HCMV seroprevalence

rate increased from 27% in children 7 months of age to 41% in children 8 years of age in a cohort of children followed for 8 years (Aarnisalo *et al.*, 2003). In a population survey in Parma, Italy, age-specific HCMV seroprevalence increased from 28% in two year olds to 96% in 45–54-year-old residents (Natali *et al.*, 1997). Similiarly, in Spain, the CMV seroprevalence rate in children 2 to 5 years of age was 42% increasing to 79% in adults 31 to 40 years of age (de Ory *et al.*, 2004). Recent studies in blood donors have demonstrated that populations in Asia and Africa continue to have CMV seropositivity rates of 95%–100% (Urwijitaroon *et al.*, 1993; Lu *et al.*, 1999; Pultoo *et al.*, 2001; Kothari *et al.*, 2002) whereas in Germany the HCMV seropositivity rates in blood donors are lower ranging from 30% in 18 to 20 year olds to >70% in adults >65 years of age (Hecker *et al.*, 2004).

Although the exact mode of HCMV acquisition is unknown, it is assumed to be through direct contact with body fluids from an infected person. The differences in age-related prevalence probably reflect differences in child rearing practices, sexual behaviors, and possibly, living conditions. Breastfeeding, group care of children, crowded living conditions, and sexual activity have all been associated with high rates of HCMV infections. Sources of virus include oropharyngeal secretions, urine, cervical and vaginal secretions, semen, breast milk, blood products, and allografts (Hayes *et al.*, 1972; Reynolds *et al.*, 1973; Lang, 1975; Alford *et al.*, 1980). Presumably, exposure to saliva and other body fluids containing infectious virus is a primary mode of spread and because infected infants typically excrete significant amounts of HCMV for months to years following infection. Even older children and adults shed virus for prolonged periods (>6 months) following a primary HCMV infection. In addition, a significant proportion of seropositive individuals continue to shed virus intermittently.

An important determinant of the frequency of congenital and perinatal HCMV infections is the seroprevalence rate in women of child-bearing age. The incidence of congenital HCMV infection is directly related to the seroprevalence rates. Studies from United States and Europe have shown that the seropositivity rates in young women range from less than 50% to 85% (Krech *et al.*, 1971; Gold and Nankervis, 1982). In contrast, most women of child bearing age in less well developed regions are HCMV antibody positive (Schopfer *et al.*, 1978; Stagno *et al.*, 1982; Vial *et al.*, 1985). Prospective studies of pregnant women in the United States have shown that the rate of HCMV acquisition in young women of lower income is about 6% per year compared with about 2% in women of middle to upper income background (Stagno *et al.*, 1986).

Perinatal HCMV acquisition, including congenital infection contributes significantly to the spread of HCMV in the population because infected infants excrete large amounts of virus for prolonged periods of time. An additional and less well appreciated mode of virus spread is through breast milk. It is estimated that over 80% of breast-fed infants of persistently infected mothers will be exposed to HCMV as a result of breast feeding (Hayes *et al.*, 1972; Stagno *et al.*, 1980). Similar to congenital infections, infants infected through breast feeding will excrete virus for prolonged periods of time, making them ideal vectors for the spread of virus. Children continue to acquire HCMV infection throughout childhood and the rate of infection continues to increase during adolescence and early adulthood secondary to sexual exposure. Significant titers of infectious HCMV can be found in semen and cervical secretions, suggesting that exposure to the body fluids could result in the transmission of HCMV (Jordan *et al.*, 1973; Willmott, 1975; Drew *et al.*, 1981; Chandler *et al.*, 1985a,b). The natural history of HCMV infection in adolescents and adults has been shown to parallel sexually transmitted diseases (STDs) (Knox *et al.*, 1979; Sohn *et al.*, 1991). Homosexual men and women attending STD clinics have an increased incidence of HCMV infection (Drew *et al.*, 1981). Thus, HCMV should be considered an STD in adults that can effectively spread through a sexually active population (Table 44.1).

Transmission of HCMV by mothers to infants: perinatal infections

Studies of mothers and infants from various countries in previous decades suggest that HCMV infections in young infants are acquired from their mothers (Numazaki *et al.*, 1970; Hayes *et al.*, 1972; Reynolds *et al.*, 1973; Granstrom and Leinikki, 1978; Alford *et al.*, 1980; Stagno *et al.*, 1980; Dworsky *et al.*, 1983a,b). HCMV may be transmitted from the mother to the infant either through the genital tract at delivery or through breast milk (Hayes *et al.*, 1972; Reynolds *et al.*, 1973; Stagno *et al.*, 1975; Alford *et al.*, 1980; Stagno *et al.*, 1980; Dworsky *et al.*, 1983a,b). As seen in Table 44.2, the rates of HCMV excretion in infants differ by country (Stagno *et al.*, 1980). Children from countries such as Japan, Thailand, and Guatemala, where the practice of breast-feeding is almost universal and the majority of women of childbearing age are seroimmune to HCMV, have higher rates of HCMV excretion during the first year of life than infants from other countries where breast-feeding is less common (Stagno *et al.*, 1980; Stagno and Cloud, 1990). (Table 44.2)

Table 44.1. Sources and routes of transmission of HCMV infection

	Mode of exposure and transmission
Community acquired, age	
Perinatal	Intrauterine fetal infection (congenital); intrapartum exposure to virus; breast milk acquired; mother-to-infant transmission
Infancy and childhood	Exposure to saliva and other body fluids; child-to-child transmission
Adolescence and adulthood	Exposure to young children; sexual transmission; possible occupational exposures
Hospital acquired, source	
Blood products	Blood products from seropositive donors; multiple transfusions; white blood cell containing blood products
Allografts	Allograft from seropositive donors
Donor semen	Artificial insemination using semen from seropositive donors

Breastfeeding practices have a major influence on the epidemiology of postnatal HCMV infections (Stagno and Cloud, 1994; Bryant *et al.*, 2002). HCMV isolation from breast milk was first described over 35 years ago by Diosi *et al.,* (1967). The importance of breast milk as a source of HCMV was first recognized by Hayes *et al.* (1972). HCMV has been detected in breast milk in 13% to 50% of lactating women using conventional virus isolation techniques (Yeager, 1975; Stagno *et al.*, 1980; Dworsky *et al.*, 1983). More recent studies utilizing the more sensitive PCR technology demonstrated the presence of HCMV DNA in breast milk from >90% of seropositive women (Hamprecht *et al.*, 1998, 2001; Maschmann *et al.*, 2001). The mechanisms of HCMV reactivation and excretion of HCMV in breast milk have not been defined. The early appearance of viral DNA in milk whey, the presence of infectious virus in milk whey, and higher viral load in breast milk have been shown to be risk factors for transmission of HCMV infection (Hamprecht *et al.*, 1998, 2001; van der Strate *et al.*, 2001).

In term infants, the consequences of HCMV infection acquired via breast milk has been reported to be negligible (Hayes *et al.*, 1972; Stagno *et al.*, 1980; Dworsky *et al.*, 1983). In contrast, postnatal HCMV infection can lead to symptomatic infection in about 10% to 50% of preterm infants leading to significant morbidity (Alford *et al.*, 1980; Dworsky *et al.*, 1983; Yeager, 1983; Paryani *et al.*, 1985; Hamprecht *et al.*, 1998, 2001). A prospective study of 41 seropositive mothers and their infants revealed that 12/31 infants who were breastfed for >1 month acquired HCMV infection compared with none of the 10 infants who were breastfed for <1 month (Dworsky *et al.*, 1983). Although it is clear that postnatal HCMV infection acquired through breast milk causes symptomatic infection such as sepsis-like syndrome in preterm infants, there is conflicting data on the occurrence of long-term sequelae. The association between postnatal HCMV infection and adverse neurodevelopmental outcome in preterm infants was reported in an earlier study by Paryani *et al.,* (1985). However, a more recent prospective study demonstrated that none of the 22 preterm infants with early postnatally acquired HCMV infection developed hearing loss or other neurologic sequelae (Vollmer *et al.*, 2004).

Table 44.2. Urinary HCMV excretion rates during the first two years of life[a]

Country	Total number of infants	% excreting HCMV by age							
		At birth	1 m	2 m	3 m	6 m	9 m	12 m	24 m
Japan	257	–	6	10	20	56	44	22	7
Thailand	140	–	–	–	38	55	18	15	–
Guatemala	109	–	–	–	23	42	40	35	–
Finland	105	2.3	–	12	23	35	25	33	41
Finland	148	2	–	16	32	36	–	39	–
Sweden	326	1	12	–	–	–	–	23	–
US, Seattle	92	1	3	–	11	13	–	11	–
UK	118	2.5	–	–	–	9	–	–	–
UK	1395	0.4	–	1.8	3.2	–	5.8	–	4
US, Birmingham	154	1.3	2	4	7	8	8	8	9

[a]Table used by permission (Stagno *et al.*, 1980).

Children-to-children transmission of HCMV

Young children are a known source of HCMV infection in the population. After early infancy where mother-to-child transmission occurs, young children likely acquire HCMV through horizontal transmission from other children or possibly indirectly through environmental contamination. Children who do not attend day-care centers have rates of HCMV seropositivity that remain generally stable until school age (Alford *et al.*, 1980; Yow *et al.*, 1987; Stagno and Cloud, 1994). However, studies in day-care centers

Table 44.3. HCMV excretion among children in day-care centers

Author	Year	Study location	% (number excreting virus/number of children)[a]
Strangert	1976	Sweden	35 (7/20)
Strom	1979	Sweden	72 (13/18)
Pass	1984	US, Birmingham	57 (59/103)
Adler	1985	US, Richmond	24 (16/66)
Hutto	1985	US, Birmingham	41 (77/188)
Jones	1985	US, San Francisco	22 (31/140)
Murph	1986	US, Iowa City	21 (17/80)
Grillner	1986	Sweden	27 (16/60)
Nelson	1987	UK	27 (32/117)
Adler	1988	US, Richmond	53 (55/104)
Volpi	1988	Italy	13 (33/253)
de Mello	1996	Brazil	52 (31/60)
Ford-Jones	1996	Canada	17 (79/471)
Kashiwagi	1999	Japan	22 (12/54)

[a]Virus excretion either from saliva or urine or both.

throughout the world have demonstrated that young children shed virus in saliva and urine creating exposure opportunities for virus transmission to other children in the day-care setting, to their parents and to the day-care or nursery workers. As illustrated in Table 44.3, HCMV excretion is common in children who attend day-care centers, although the percentage of children excreting virus varies by location (Strangert *et al.*, 1976; Strom, 1979; Pass *et al.*, Adler, Hutto *et al.*, 1985; Jones *et al.*, 1985; Grillner and Strangert, 1986; Murph *et al.*, 1986; Nelson *et al.*, 1987; Adler, 1988a,b; Volpi *et al.*, 1988; de Mello *et al.*, 1996; Ford-Jones *et al.*, 1996; Kashiwagi *et al.*, 2001) (Table 44.3).

Prospective studies in day-care centers have provided evidence of child-to-child transmission by documenting acquisition of HCMV in children previously known to be uninfected upon enrollment in the day care centers (Pass *et al.*, 1984; Adler, 1988a,b; de Mello *et al.*, 1996). Pass *et al.* followed a cohort of children <12 months of age in a day care center and found that <10% were shedding virus at enrollment but 6 to 12 months later 78% of the children in the cohort were shedding virus (Pass *et al.*, 1984). Similarly, Adler followed a group of children for 26 months in a day-care center in Richmond, Virginia and found that virus excretion increased from 25 to 61% in children <3 years of age (Adler, 1988a,b). A study in a day-care center in Sao Paulo, Brasil identified 37 children who were initially HCMV seronegative upon enrollment into the study and demonstrated that 6 to 12 months later, 50% of these children excreted HCMV in either their saliva or urine (de Mello *et al.*, 1996).

Studies that have provided appropriate control groups for comparing the day-care center populations have found that the prevalence of HCMV excretion is significantly higher in the children who attend a day-care center than in the control children who did not attend a day-care center (Hutto *et al.*, 1985; Adler, 1988a,b). In both Richmond, Virginia (odds ratio (OR) = 4.3, 95% confidence interval (CI), 2.2–8.1) and Birmingham, Alabama (OR = 3.9, 95% CI, 1.8–9.0), children attending day-care centers were approximately four times more likely to shed HCMV than children who were not enrolled in day-care centers (Adler, 1985; Hutto *et al.*, 1985).

Besides child-to-child transmission, HCMV is also found on toys and other environmental surfaces in day-cares providing another viral source for HCMV infection (Faix, 1985; Hutto *et al.*, 1986; Schopfer *et al.*, 1986). Although HCMV is usually considered labile under most environmental conditions, a study led by Hutto, found that HCMV survived on toys for up to 30 minutes in a day-care where children aged six to 30 months of age were placing a hand or toy in their mouths every one to two minutes (Hutto *et al.*, 1986). Likely horizontal transmission of HCMV from child to child occurs through saliva on hands and toys. As seen in Fig. 44.1, the highest rates of HCMV excretion in day-care settings are usually seen among toddlers and young children (ages 12–24 months and 25–36 months) supporting the theory that HCMV transmission occurs through saliva on hands and toys as children play together (Strangert *et al.*, 1976; Strom, 1979; Pass *et al.*, 1984; Adler *et al.*, 1985; Jones *et al.*, 1985; Murph *et al.*, 1986; Ford-Jones *et al.*, 1996; Kashiwagi *et al.*, 2001). (Fig. 44.1)

The strongest evidence to support child-to-child transmission of HCMV has been obtained by the analysis of the restriction endonuclease digestion patterns of HCMV DNA of isolates obtained from HCMV infected children attending day-care centers. Adler examined the restriction endonuclease patterns of HCMV isolates obtained from 16 children at a single day-care center and found that one group of seven children and another group of four children were excreting identical strains (Adler, 1988a,b). The seven children in one group were all <29 months of age and all but one of these children shared the same classroom. The four children in the second group were >36 months of age and three of the children were in the same room. In another prospective study by Adler, 104 children from a day-care center were followed and 14 different strains of HCMV were identified by restriction endonuclease analysis (Adler, 1988). Three of the 14 strains infected 44 of the children attending the day-care center. All children infected with one of the three strains of HCMV were younger than 3 years of age indicating frequent child-to-child transmission of

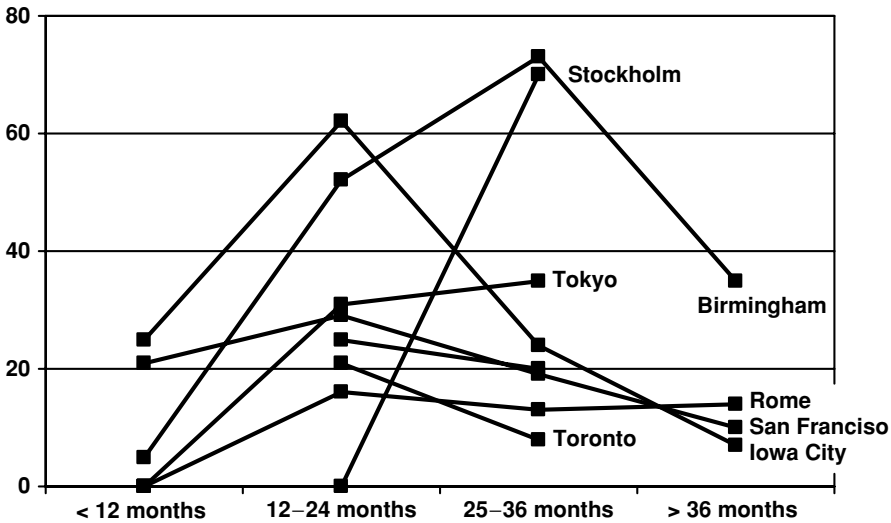

Fig. 44.1. Age-related HCMV excretion in various day-care center populations.

HCMV in these young children within the day-care setting. Other studies using restriction endonuclease analysis of HCMV DNA in different populations have also found identical HCMV strains are shared among children in group day-care confirming that HCMV is efficiently transmitted from child to child in day-care settings (Grillner and Strangert 1986; Murph *et al.*, 1991).

The higher HCMV excretion rates in young children attending day-care centers ranging from 13% to 72% compared to children who do not attend day care; the highest rates of HCMV excretion observed in toddlers and young children; the documentation of HCMV shedding in children previously known to be uninfected after day-care center attendance in prospective studies; the isolation of HCMV from toys and other objects in day-care settings; and the identification of similar strains of HCMV within day-care centers provides compelling evidence for child-to-child transmission among young children in day-care settings.

Transmission of HCMV by children to parents

Children excreting virus often become the source of HCMV infection for susceptible parents and others in the household. Studies by Spector *et al.*, and Dworsky *et al.*, using restriction endonuclease analysis, have demonstrated that infants with perinatal HCMV infections may transmit HCMV to an uninfected parent or other adult family member (Spector and Spector, 1982; Dworsky *et al.*, 1984). Seroconversion rates for parents when children reside in the household are higher than the rates reported for households with only adults in residence. In a study by Yeager *et al.*, infants who were nosocomially infected with HCMV by transfusion transmitted HCMV infection to their seronegative mothers (Yeager, 1975). Of the mothers who were seronegative and exposed to infected infants, 47% (7/15) seroconverted within a year after the child was discharged from the hospital compared to 4% of the mothers whose infants were uninfected at the time of discharge. Dworsky *et al.* studied 372 women between pregnancies and observed these women had an annual seroconversion rate of 5.5% compared to 2.2% for women who were pregnant for the first time (Dworsky *et al.*, 1983). Taber *et al.*, in a family HCMV study, demonstrated an increased seroconversion rate within the family setting (Taber *et al.*, 1985). The annual seroconversion rate for parents with young children in the household was 10.6% for mothers and 10% for fathers in this study. In 10 of 14 families where the initial family member who seroconverted could be identified, the index case was a child. In another study by Pass *et al.*, virus isolates were collected from the members of five families where the mother had evidence of a primary maternal infection during her most recent pregnancy and a child less than three years of age who was excreting HCMV residing in the home (Pass *et al.*, 1987). In each of the five families, the HCMV strains were identical by restriction endonuclease analysis. In two families, acquisition of HCMV by the child in a day-care center was followed by both maternal seroconversion and maternal excretion of the same identical strain of HCMV as shed by her child. This study provides further evidence that children in the household, especially

Table 44.4. Risk factors for primary HCMV infection in women attending an STD clinic (Chandler. *et al.*, 1985a,b)

Factor	Seroconversion (11/84, 13%)	Remained seronegative (15/84, 18%)	*P* value
Age (years)	20.4 ± 0.9	22.3 ± 0.5	NS
>two recent sexual partners	67%	14%	0.01
History of STDs	73%	32%	0.02
STDs during study	73%	63%	NS

those attending group day-care may transmit HCMV to other family members.

Other studies in day-care centers have provided compelling evidence of the acquisition of HCMV by children in day care with the subsequent seroconversion of their parents (Adler, 1985; Pass *et al.*, 1986; Adler, 1991). In a longitudinal follow-up study of seronegative parents with children in day-care centers and a control group of parents whose children were not in day-care centers, 21% (14/67) of the parents whose children were in the day-care centers seroconverted whereas none (0/31) of the controls seroconverted (Pass *et al.*, 1986). All of the parents who seroconverted had a child who was shedding HCMV either in saliva or urine. Parents at greatest risk of HCMV infection were the parents of children who were found to be shedding HCMV (45%, 9/20) and were <18 months of age at enrollment. In a similar study, Adler observed that 39% (7/18) of mothers seroconverted within 3 to 7 months after their children became infected at the day-care center (Adler, 1988a,b). The HCMV strains isolated were identical in both the mothers and their children, and in six of the mothers the HCMV strain was associated with the day-care center. An additional study from Adler prospectively followed 96 seronegative mothers of children attending three day-care centers (Adler, 1991). Among the 50 mothers with HCMV infected children, 19 seroconverted (38%) whereas, only 2 of the 46 seronegative mothers of uninfected children seroconverted (4%). Of the 19 mothers who seroconverted, 9 shed HCMV and the virus was an identical isolate as was shed by their child. All of these studies provide evidence that young children contribute to parental HCMV infections and mothers of these young children are at an increased risk of HCMV infection that might result in congenital infections for their future offspring (Pass *et al.*, 1987).

HCMV transmission through sexual activity

An important source of HCMV infection is through the intimate contact with oral and genital secretions (Britt and Alford, 1996). Salivary glands are a site of virus persistence in humans and it is likely that reactivations leads to the presence of infectious virus in oral secretions. Infectious virus can often be cultured from cervical secretions and semen has been shown to be a rich source of virus in seropositive men (Lang, 1975; Knox *et al.*, 1979; Stagno *et al.*, 1975). High rates of HCMV seropositivity have been reported in women attending STD clinics and couples discordant for HCMV have been shown to readily transmit virus and in some cases, reinfect previously infected partners (Handsfield *et al.*, 1985; Chandler *et al.*, 1985a,b, 1987).

Chandler *et al.*, examined the association between HCMV infection and indices of sexual activity in 347 women attending an STD clinic (Chandler *et al.*, 1985a,b). To determine the annual incidence of primary HCMV infection, 84 seronegative women were followed for a mean duration of 18.4 weeks with documented seroconversion in 11 (13%). Based on this study, the annualized incidence of primary HCMV infection was estimated to be 37% (Table 44.4). As can be seen in Table 44.4, the risk factors acquisition of HCMV infection in seronegative women in this population include the history of STDs and >2 sexual partners in the month preceding seroconversion. However, the majority of subjects included in the study were selected based on the presence of mucopurulent cervical discharge or cervical ectopy which may have resulted in the inclusion of more women with STDs in the study. In a more recent study from Seattle by Coonrod *et al.*, 245 HCMV seronegative women attending an STD clinic were followed for a median duration of 23 months. During the study, seroconversion was documented in 36 (15%) women, an annualized rate of 10%–12% (Coonrod *et al.*, 1998). The risk factors that were associated with seroconversion in that study include young age, younger age at sexual debut, greater number of sexual partners, and more recent sexual partners. In addition, seroconverters were more likely to have gonorrhea, chlamydia or pelvic inflammatory disease. (Table 44.4)

The shedding of HCMV in genital tract secretions was examined in studies of selected populations (Pereira *et al.*, 1990; Fairfax *et al.*, 1994; Shen *et al.*, 1994; Clarke *et al.*, 1996). Collier *et al.*, studied the association between HCMV shedding and sexual activity in 1481 women attending an STD clinic (Collier *et al.*, 1995). HCMV was isolated in cervical secretions from 9.4% of the 951 seropositive women. Cervical HCMV excretion was associated with concomitant gonococcal infection and was less frequent in women using barrier contraception. Higher frequency of HCMV-IgM antibodies were observed in seroconverters, suggesting recent HCMV infection and possible exogenous reinfection. The frequency and the factors associated with cervical shedding of HCMV were compared between a group of 195 licensed prostitutes and 187 women attending an

STD clinic in Taiwan (Shen *et al.*, 1994). About a third of the women in both groups shed HCMV in cervical secretions. The factors that were associated with cervical HCMV shedding are multiple sexual partners, and history of STDs. In another study of STD clinic attendees by Pereira *et al.*, age less than 23 years and concomitant gonococcal infection were independently associated with cervical HCMV excretion (Pereira *et al.*, 1990). Women with another genital tract infection were 6.5 times more likely to have HCMV than those without other genital infections. In a more recent study by Clarke et al., HCMV shedding in HIV seronegative and HIV seropositive women from an urban minority community was examined (Clarke *et al.*, 1996). HCMV seropositivity was >90% in both groups. Cervicovaginal shedding was detected in 4.4% of HIV-negative women and 19.6% of HIV-positive subjects (odds ratio, 5.28; $P < 0.001$). Multiple logistic regression analysis revealed that HCMV shedding was associated with younger age (OR = 0.90; $P < 0.001$), and concurrent chlamydial or gonococcal infection (OR = 3.60; $P < 0.08$). Among HIV-positive subjects, CMV shedding was significantly higher in women with CD4 cell counts $<500 \times 10^6$/L. The data from the studies described above provides evidence that cervicovaginal HCMV infection is related to sexual activity, and the presence of other genital tract infections.

Several studies have identified the risk factors for seropositivity to HCMV in women attending STD clinics, in pregnant women, sexually active adolescent girls, and HIV infected women (Chandler *et al.*, 1985a, b; Collier *et al.*, 1990; Sohn *et al.*, 1991; Shen *et al.*, 1994; Clarke *et al.*, 1996; Coonrod *et al.*, 1998). The results from these studies have been summarized in Table 44.5. In a study of 347 women attending an STD clinic, a stepwise logistic regression analysis showed that HCMV seropositivity was associated with non-white race, number of lifetime sexual partners, and young age at sexual debut (Chandler *et al.*, 1985a,b). However, the majority of women included in the study had cervical abnormalities. A latter study from the same STD clinic including unselected study subjects and larger sample size has attempted to define the relationship between sexual practices and HCMV seropositivity in African American and Caucasian women (Collier *et al.*, 1990). The risk factors associated with HCMV seroprevalence are similar in both African American and Caucasian women and these include more recent new sexual partners, more lifetime sexual partners, and the presence of chlamydial infection. Additional risk factors in Caucasian women included young age at sexual debut and not using barrier contraception. In another study from Seattle, the risk factors for the presence of HCMV antibodies were determined in 1129 pregnant women (Chandler *et al.*, 1985a,b). Fifty seven percent of women were HCMV seropositive. Logistic regression analysis showed the seropositivity correlated with lower socioeconomic status, older age, history of abnormal cervical cytology, infection with *Trichomonas vaginalis*, and greater number of sexual partners.

In a study of 254 adolescent girls attending a contraceptive counseling clinic, Sohn *et al.*, demonstrated a strong association between indicators of sexual activity and the presence of CMV-IgG antibodies (Sohn *et al.*, 1991). Using logistic regression analysis, the presence of two more sexual activity risk factors (young age at sexual debut, years of sexual activity, number of lifetime partners) was the most important predictor of HCMV infection. After controlling for confounders, African-American race was associated

Table 44.5. Risk factors for CMV seropositivity in adolescents, contraceptive and STD clinics

Author and Year published, location	Study population	Risk factors for CMV seropositivity
Chandler, 1985 Seattle, US	347 STD clinic clients (219 with cervical abnormalities	Young age sexual debut More lifetime sexual partners More recent sexual partners
Collier, 1990 Seattle. US	1481 STD clinic clients	*African Americans*: Recent sexual partners New sexual partners Cervical chlamydial infection *Caucasians*: Young sexual debut Recent new sexual partners More lifetime sexual partners Lack of barrier contraception
Sohn, 1991 Birmingham, US	254 adolescent girls (12–18 years)	African American race STDs >2 sexual partners >3 years of sexual activity
Hyams, 1993 Philippines	470 male military personnel presenting at a STD clinic	History of STDs
Shen, 1994 Taiwan	195 licensed prostitutes 187 STD clinic clients 70 women attending a gynecologic clinic	Presence of cervical CMV shedding
Ray, 1997 India	368 in STD and antenatal clinics	Chlamydial endocervicitis
Rosenthal, 1997 Cincinnati, US	399 adolescents	African American race Female sex
Coonrod, 1998 Seattle, US	245 STD clinic clients	More sexual partners New sexual partners Gonorrhea Chlamydia Pelvic inflammatory disease

with an increased risk of infection (OR = 3.4) whereas oral contraceptive use was protective (OR = 0.6) for HCMV infection. The association between sexual activity and the HCMV seropositivity has also been documented in other studies from different geographic regions (Table 44.5) (Berry *et al.*, 1988; Pereira *et al.*, 1990; Hyams *et al.*, 1993; Shen *et al.*, 1994; Coonrod *et al.*, 1998). (Table 44.5)

Further support for the sexual transmission of HCMV was provided by a study of HCMV infection in sexual partners by Handsfield *et al.* (1985). This study demonstrated that 74% of men whose female partners were seropositive were antibody positive compared to 34% of those whose partners were seronegative ($P = 0.008$). Restriction endonuclease analysis of HCMV isolates from three pairs of sexual partners identified that two of the couples were infected with common strains. To determine the frequency of HCMV reinfection, Chandler et al. examined serial isolates from eight women attending an STD clinic and seven women receiving routine prenatal care (Chandler *et al.*, 1987). Using restriction digestion analysis, the authors demonstrated that four of the eight women from the STD clinic were infected with more than one virus strain. Two women shed different strains in serial samples, and two women shed different strains simultaneously from different body sites. These findings provide evidence that reinfection with different virus strains is not uncommon in women with increased exposure to HCMV.

The association between sexual activity and HCMV transmission can be summarized as follows: (a) the prevalence of HCMV antibody more than doubles during the years beginning sexual activity (15–30 yrs) (Wentworth and Alexander, 1971), (b) higher rates of seropositivity in male partners of seropositive women as compared to seronegative women (Numazaki *et al.*, 1970; Handsfield *et al.*, 1985), (c) HCMV has been isolated from cervix of 13% to 35% of women with suspected STDs (Knox *et al.*, 1979; Shen *et al.*, 1994), (d) seropositivity correlated with the presence of other STDs (Chandler *et al.*, 1985a,b; Collier *et al.*, 1995; Coonrod *et al.*, 1998), (e) among seronegative women attending an STD clinic, the annual HCMV seroconversion rate was noted to be 37% vs. 1% to 2% per year in the general population (Table 44.5) (Chandler *et al.*, 1985a,b), (f) seropositivity correlated with number of lifetime sexual partners and young age at onset of sexual activity (Collier *et al.*, 1990; Pereira *et al.*, 1990; Shen *et al.*, 1992) and, (g) a negative correlation between the use of barrier contraception and seropositivity (Collier *et al.*, 1990, 1995).

Thus, there is strong epidemiological evidence that acquisition and transmission of HCMV infection is associated with sexual activity and STDs. However, the mechanisms and pathogenesis of this association have not been defined. Although frequent and repeated exposure is an important determinant for HCMV infection in individuals with STDs, it is also possible that the presence of genital tract inflammation plays an important role by providing a local milieu that is conducive to HCMV replication and transmission. However, the relationship between genital tract inflammation and the local HCMV replication has not been studied.

Transmission of HCMV to child-care providers

Children in day-care settings may be an important source of HCMV infection for child-care personnel. Numerous studies in the past decades have described the risk of HCMV infection for women who provide care for children in an occupational setting, as seen in Table 44.6 (Jones *et al.* 1985; Blackman *et al.*, 1987; Nelson *et al.*, 1987; Adler, 1989; Pass *et al.*, 1990; Murph *et al.*, 1991; Ford-Jones *et al.*, 1996; Bale *et al.*, 1999; De Schryver *et al.*, 1999). Overall, the annual seroconversion rates in day-care workers who care for young children range from 0% to 20%. Variations in seroconversion rates within differing populations may reflect underlying factors such as socioeconomic status and race and their impact on HCMV infection. The populations with the highest seroconversion rates were those that studied day-care providers in various regions of the US and Canada (Adler, 1989; Pass *et al.*, 1990; Murph *et al.*, 1991; Ford-Jones *et al.*, 1996; Bale *et al.*, 1999). Studies in England and Belgium found lower seroconversion rates among individuals who were exposed to children in occupational settings (Nelson *et al.*, 1987; De Schryver *et al.*, 1999). This lower transmission rate may be due to the smaller sample sizes and the shorter follow up periods for the detection of seroconversion. It is also possible that lower levels of virus shedding or the virologic characteristics including the infectivity of the circulating HCMV strains might account for the lower rates of transmission. Another explanation could be that child care personnel in these countries are better educated and adhere to standard hygiene practices when caring for young children thereby interrupting the transmission of the virus. Although HCMV seroconversion rates in child care providers vary in different populations and studies, the higher seroconversion rates observed in the larger cohort studies provide evidence that in some populations, young children play an important role in transmitting HCMV to their care providers (Adler, 1989; Pass *et al.*, 1990; Murph *et al.*, 1991; Ford-Jones *et al.*, 1996; Bale *et al.*, 1999). (Table 44.6).

Table 44.6. Annual HCMV seroconversion rates among child care personnel

Author, year, and location	Population	HCMV seropositivity, %	Annual seroconversion rate, %
Jones, 1985 US, San Francisco	130 infant development center workers	50	0
Blackman, 1987 US, Iowa City	57 preschool workers for physical and mentally impaired children program	35	0
	53 home-based early intervention program workers	40	7.7
	66 hospital clinic staff	38	0
Nelson, 1987 England	41 day-care teachers	66	0
	500 matched controls in their first pregnancy	53	NR[a]
Adler, 1989 US, Richmond	610 day-care workers	41	11
	565 hospital workers	47	2.2
Pass, 1990 US, Birmingham	509 day-care workers	62	20
Murph, 1991 US, Iowa City	252 day-care workers	38	7.9
Ford-Jones, 1996 Canada	206 day-care worlers	67	12.5
Bale, 1999 US, Iowa City	132 women providing child care in their homes	58	6.8
de Schryver, 1999 Belgium	283 educators for mentally disabled children	15.9	1.03
	294 nurses for the elderly	18.4	1.42

[a]NR = Not reported.

Risk factors for HCMV serconversion of child care providers have included workers <30 years of age, not wearing gloves when changing diapers, and caring for children ≤3 years of age for 20 hours a week (Pass *et al.*, 1990; Ford-Jones *et al.*, 1996). However, other studies did not find that seroconversion of day care providers was related to caring for children ≤3 years age (Adler, 1989; Murph *et al.*, 1991; Ford-Jones *et al.*, 1996). None of the studies found that race or the presence of other children in the home were associated with seroconversion in an occupational setting (Adler *et al.*, 1990; Murph *et al.*, 1991; Ford-Jones *et al.*, 1996). In several studies where day care children were evaluated for virus shedding, similar viral isolates were found in both the children and in the day-care providers who seroconverted suggesting that HCMV infection in the workers were acquired from the children in their care (Adler, 1989; Murph *et al.*, 1991; Bale *et al.*, 1999).

Seroprevalence studies of child care personnel have identified factors associated with HCMV seropositivity in these workers as illustrated in Table 44.7 (Blackman *et al.*, 1987; Volpi *et al.*, 1988; Adler, 1989; Pass *et al.*, 1990; Murph *et al.*, 1991; Ford-Jones *et al.*, 1996; Jackson *et al.*, 1996; Bale *et al.*, 1999; De Schryver *et al.*, 1999; Kiss *et al.*, 2002). The risk factors observed for HCMV seropositivity include factors that are associated with HCMV in the general population such as parity, age and race (Chandler *et al.*, 1985a,b; Collier *et al.*, 1990; Sohn *et al.*, 1991) and also additional risk factors related to occupation as a child-care provider. These occupational risk factors suggest that HCMV infection in occupational settings may result from the exposure to young children who are shedding virus. However, none of these studies can provide with certainty that all HCMV infections observed in the day-care personnel are due to child-to-worker transmission since other HCMV exposure opportunities may exist in the environment and the population from other sources. (Table 44.7)

Transmission of CMV in health-care settings

The possibility that CMV may be transmitted in health care settings has been explored over the last three decades. In 1969, Haldane *et al.*, reported a significantly higher incidence of birth defects among the offspring of nurses caring for children with CMV infection (Haldane *et al.*, 1969). Following this report, many studies (Table 44.8) have evaluated the risk of CMV infection in nurses who cared for young infants and children in the newborn nurseries or pediatric wards of hospitals and provided conflicting results (Yeager, 1975; Haneberg *et al.*, 1980; Ahlfors *et al.*, 1981; Dworsky *et al.*, 1983a,b; Friedman *et al.*, 1984; Hatherley, 1985; Adler *et al.*, 1986; Balfour and Balfour, 1986; Hatherley, 1986; Demmler *et al.*, 1987). Overall, the studies with appropriate control groups found the combined risk of CMV infection in nurses who cared for children to be almost three times (risk ratio (RR) 2.7, 95% CI, 1.3 – 5.5) greater than the controls (Yeager, 1975; Ahlfors *et al.*, 1981; Dworsky *et al.*, 1983; Friedman *et al.*, 1984; Balfour and Balfour, 1986; Hatherley,

Table 44.7. Risk factors for HCMV seropositivity among child care personnel

Author, year, and location	Population	HCMV seropositivity, %	Risk factors
Blackman, 1987 Iowa City, US	57 preschool workers for physical and mentally impaired children program	35	Older age
	53 home-based early intervention program workers	40	
	66 hospital clinic staff	38	
Volpi, 1988 Italy	82 day-care workers	96	Multiparous[a]
	82 housewife controls	84	
	229 female day-care students in training	85	
Adler, 1989 Richmond, US	610 day-care workers	41	Cared for children <2 years of age
	565 hospital workers	47	
Pass, 1990 Birmingham, US	509 day-care workers	62	Older age Non-white race Working with children <2 years of age for 8 hours/week
Murph, 1991 Iowa City, US	252 day-care workers	38	Older age Non-white race Children residing in home
Ford-Jones, 1996 Canada	206 day-care worlers	67	Born outside of Canada Children <5 residing in house Household size >3 people
Jackson, 1996 Seattle, US	360 child care workers	62	Non-white race Changing diapers ≥3 days/week at work Children residing in house
Bale, 1999 Iowa City, US	132 women providing child care in their homes	58	Caring for toddlers (1–2 yrs) Longer time as a child care provider
de Schryver, 1999 Belgium	283 educators for mentally disabled children	16	Muliparous
	294 nurses for the elderly	18	
Kiss, 2002 Belgium	211 kindergarten teachers	29%[b]	Children residing in house
	283 administrative workers		Kindergarten teaching

[a]Borderline significance.
[b]Reported for the combined groups.

1986; Flowers *et al.*, 1988). However, when person–year analysis was performed to take into account the follow-up period, a trend toward an increased risk in nurses remained but failed to reach statistical significance (RR 1.8, 95% CI, 0.9 – 3.6) (Flowers *et al.*, 1988).

Studies of health-care personnel including staff in children's hospitals have shown that CMV seroconversion rates do not differ between the employees, and the general population (Tolkoff-Rubin *et al.*, 1978; Dworsky *et al.*, 1983; Balfour and Balfour 1986; Demmler *et al.*, 1987; Balcarek *et al.*, 1990; Gerberding, 1994). However, all of the studies of health care personnel have low sample sizes, inadequate statistical power and have not provided information on potential confounding factors such as CMV sexual exposure or exposure to children outside of the workplace that could impact their risk for HCMV infection (Flowers *et al.*, 1988; Farr and Torner, 1990). These limitations and also the widespread adoption of universal precautions (CDC, 1987, 1988) in health-care settings in the late 1980s have made it difficult to estimate whether an increased risk of CMV infection exists within occupational settings. As illustrated in Table 44.9, several studies have evaluated risk factors for CMV seropositivity in health-care providers although these studies did not account for other possible non-occupational CMV exposures (Gerberding, 1994; Herbert *et al.*, 1995; Sobaszek *et al.*, 2000). Studies using molecular epidemiologic techniques demonstrated that health care workers are at low risk for occupational CMV transmission (Dworsky *et al.*, 1983; Adler, 1986; Peckham *et al.*, 1986; Demmler *et al.*, 1987). In these studies, molecular analysis of CMV isolates demonstrated that some of the CMV infections identified in the workers were similar to CMV isolates from other family members and were not occupationally acquired (Dworsky *et al.*, 1983; Demmler *et al.*, 1987). Without a large collaborative study or the pooling of results of multiple well-designed studies, the question about the relative contribution of CMV infection in health-care settings cannot be fully elucidated at this time. However,

Table 44.8. Seroconversion rates for personnel in health care settings

Study, year published, and location	Study groups	CMV seroconversion rates % (number/total)
Yeager 1975	Pediatric ward nurses	9.7 (3/31)
Denver, US	Nursery nurses	5.9 (2/34)
	Controls	0 (0/27)
Tolkoff-Rubin, 1978	Hemodialysis staff	0 (0/26)
Boston, US	Hemodialysis patients	13 (10/80)
Haneberg, 1980 Norway	Pediatric student nurses	9.4 (6/64)
Ahlfors, 1981	Pediatric ward nurses	6.9 (2/29)
Sweden	Pediatric nurses	0 (0/31)
	Controls	1.9 (1/52)
Dworsky, 1983	Nursery nurses	3.3 (4/61)
Birmingham, US	Controls (pregnant women)	1.5 (23/1549)
	Medical students	0.6 (1/89)
	Medical house staff	2.7 (1/25)
Friedman, 1984	Pediatric intensive care nurses	8.7 (2/23)
Philadelphia, US	Nursery nurses	7.7 (3/39)
	Pediatric ward nurses	4.6 (3/65)
	Controls	2.9 (1/35)
Hatherley, 1985 Australia	Nursery nurses	4.4 (2/45)
Hatherley, 1986	Nursery nurses	1.9 (3/154)
Australia	Controls	0 (0/12)
Adler, 1986	Pediatric ward nurses	6.5 (2/31)
Richmond, US	Nursery nurses	2.5 (1/40)
Balfour, 1986	Neonatal intensive care nurses	4.2 (4/96)
Minneapolis, US	Student nurses	5.0 (7/139)
	Renal transplant/hemodialysis nurses	1.7 (2/117)
	Controls	1.8 (3/167)
Brady, 1985	Nursery nurses	6.0 (4/67)
Houston, US	Medical house staff	9.5 (2/21)
Demmler, 1987	Nursery nurses	5.4 (2/37)
Houston, US	Pediatric chronic care ward nurses	0 (0/21)
	Pediatric chronic care ward therapists	0 (0/37)
Balcarek, 1990	Children hospital employees, patient care	4.4 (8/183)
Birmingham, US	Children hospital employees, administrative	3.6 (2/56)
		4.1 (2/49)
	Children hospital employees, support	8.3 (1/12)
	Children hospital employees, laboratory	
Gerberding, 1994 San Francisco, US	Health care providers at one hospital	6.6 (25/378)

the existing data suggest that the risk of CMV infection in a health-care setting is low and likely does not differ from the risk of CMV infection in the general population. (Tables 44.8 and 44.9).

Transfusion acquired HCMV infection

Transfusion associated HCMV infection was first described in 1966 and since that time, has been demonstrated to be a cause of significant morbidity and mortality in a wide variety of clinical circumstances (Kaariainen *et al.*, 1966; Hillyer *et al.*, 1990; Meyers, 1991). HCMV infection following transfusion has been associated with transfused red cell, platelet concentrates, and granulocyte concentrates whereas, fresh-frozen plasma and cryoprecipitates have not been reported to cause HCMV transmission (Bowden and Sayers, 1990). HCMV is believed to establish latency in the cells of myeloid lineage and the virus can be reactivated and cause disease in seropositive immunocompromized hosts. Transfusion of unscreened cellular components results in HCMV transmission in approximately 30%

Table 44.9. Risk factors for CMV seropositivity in health care workers

Study, year published, and location	Study groups	Risk factors for CMV seropositivity evaluated	Odds ratio (95% confidence interval)
Gerberding, 1994 San Francisco, US	976 health care providers at San Francisco General Hospital	Older age	1.4 (1.2–1.7)[a]
		Female	1.8 (1.3–2.5)
		Being a physician	0.4 (0.3–0.6)
		Working on an AIDS clinical unit	NS[b]
Herbert, 1995 England	81 preclinical dental students and 81 matched controls 53 clinical dental students and 53 matched controls 103 dental surgeons and 103 matched controls	Protective workwear	NS[b]
Sobaszek, 2000 France	400 female health care workers who had contact with children or immunosuppressed patients	Number of children ≥1	1.9 (1.2–3.0)
		Close contact nursing tasks	2.2 (1.4–3.3)
		Older age	1.7 (1.0–3.0)

[a] For an increase of one decade.
[b] NS, Not significant.

(range 10%–70%) in seronegative recipients, as determined by seroconversion and/or virus isolation (Wilhelm *et al.*, 1986; Preiksaitis *et al.*, 1988). The risk of transfusion associated HCMV infection is directly related the number of components transfused and the quantity of leukocytes transfused. HCMV can also be transmitted via organ or marrow grafts (Wreghitt *et al.*, 1988; Reusser *et al.*, 1991). The use of blood products from seronegative donors and using procedures which limit the quantity of leukocytes in the transfused blood have considerably reduced the risk of transfusion associated HCMV infection (Lang *et al.*, 1977; Brady *et al.*, 1984; Preiksaitis *et al.*, 1988; Miller *et al.*, 1991). These findings suggest that leukocytes are the cellular components of blood products responsible for most transfusion-associated HCMV infection.

Transfusion-associated HCMV infection can result in significant morbidity and mortality in preterm infants, allograft recipients, following cardiac surgery and other immunocompromized hosts (Yeager *et al.*, 1981; Brady *et al.*, 1984; Adler, 1985; Bowden, 1991). The rate of HCMV acquisition in preterm infants of seronegative mothers who received multiple transfusion has been shown be between 9% and 13.5% (Yeager *et al.*, 1981; Adler, 1983). The percentage of units of blood capable of transmitting HCMV has been estimated to be between 2.5% and 12.5%. A controlled trial in low birth weight HCMV seronegative infants born to HCMV-seronegative mothers compared the use of filtered and unfiltered red cell components for the prevention of transfusion associated HCMV infection (Gilbert *et al.*, 1989). Twenty-one percent (9/42) of the recipients of unscreened, unfiltered blood acquired HCMV infection compared with none of 59 infants who received unscreened, filtered components. Additional studies have confirmed the effectiveness of the leukofiltration in reducing the transmission of HCMV in other population groups including immunocompromized hosts (Eisenfeld *et al.*, 1992).

Transplantation and HCMV infection

Allografts from donors previously infected with HCMV represent a major risk factor for HCMV transmission. In all but the bone marrow allograft recipients, the transplantation of an organ from a donor previously infected with HCMV into a seronegative recipient has been shown to be the single most important risk factor for primary HCMV infection (Rubin *et al.*, 1985; Ho, 1991). In solid organ transplantation, primary infections that develop from the allograft have a more profound impact on the outcomes than infections acquired via transfusions (Falagas *et al.*, 1996). The natural history of HCMV infection in transplant recipients suggests that infection is nearly universal in those exposed, but the clinical disease is dependent on specific risk factors, in particular, the immunosuppressive regimen. As the severity of immunosuppression increases, so does the severity of HCMV disease, as evidenced by the often fatal HCMV infections that occur in bone marrow allograft recipients

(Meyers *et al.*, 1982; Winston *et al.*, 1990; Schmidt *et al.*, 1991). In renal allograft recipients following HCMV mismatched donor-recipient transplantation, disease rates as high as 70% have been described (Rubin and Colvin, 1986). Mortality rates in excess of 50% in heart/lung transplantation have limited the mismatched donor-recipient transplantation in some centers (Smyth *et al.*, 1991).

Historically, HCMV interstitial pneumonitis occurred in 10% to 30% of bone marrow transplant recipients, with mortality rates of over 80% in some studies, even after the introduction of ganciclvir (Wingard *et al.*, 1988; Winston *et al.*, 1988). However, the incidence of HCMV infection and disease in hematopoietic stem cell transplant recipients has decreased somewhat over the past 15 years due to changes in patient management. These changes include the avoidance of HCMV positive cellular blood products in susceptible, HCMV-negative recipients, and the use of prophylactic and/or pre-emptive antiviral therapy to limit HCMV reactivation and disease. Since, the advent of prophylactic and pre-emptive antiviral therapy, the onset time of HCMV pneumonia has been delayed from a median time of 44 days after transplant to between 92 and 188 days (Goodrich *et al.*, 1993; Nguyen *et al.*, 1999; de Medeiros *et al.*, 2000; Machado *et al.*, 2000; Ljungman, 2001). In a study of liver transplant recipients, despite the significant increase in the proportion of high risk patients (HCMV recipient-/donor+) and the increase in the rate of HCMV infection, the incidence of HCMV disease has decreased significantly (Singh *et al.*, 2004). The authors of that study noted that the HCMV infection rate neither confounded the use of antiviral prophylaxis nor selective testing for HCMV. In addition to the morbidity and mortality associated with HCMV disease, the survival of the allografts has been adversely affected by HCMV.

HCMV transmission from artificial insemination by donor semen

The advent of artificial insemination with donor semen suggests another possible transmission route for HCMV in the population. HCMV has been cultured from semen even in cases in which urine, saliva or blood specimens have been negative for infectious virus (Lang and Kummer, 1975; Biggar *et al.*, 1983; Mascola and Guinan, 1986; Tjiam *et al.*, 1987). It has been demonstrated that HCMV transmission can occur through therapeutic donor insemination (Prior *et al.*, 1994). However, most of the studies examining the presence of HCMV in semen have included individuals attending STD clinics. In a more recent study from France, Masat et al. examined the presence of HCMV in cryopreserved semen samples collected for therapeutic donor insemination (Mansat *et al.*, 1997). Using cell culture and PCR, HCMV was detected in 5.1% of the semen specimens suggesting that cryopreserved semen from healthy donors may represent a potential source of HCMV infection. The American Fertility Society recommended that all semen donors be screened for the presence of HCMV antibodies and that semen from seropositive donors may only be used to inseminate seropositive recipients. Although the risk to offspring via donor insemination has not been defined, the British Andrology Society recommends that only semen from seronegative donors be stored for clinical use (British Andrology Society, 1999).

Summary

HCMV remains a ubiquitous infectious agent and a cause of significant disease in individuals with immature or suppressed immune responses. In most human populations examined, HCMV is acquired predominantly through exposure to young children, breast feeding and sexual activity. Although a strong epidemiologic association between these risk factor and acquisition of HCMV has been demonstrated, the exact mechanism and pathogenesis of HCMV transmission have not been defined. Furthermore, the roles of virologic characteristics including strain variation and reinfections in the transmission of HCMV have not been delineated. Today, HCMV is primarily a cause of disease in newborn infants and in allograft recipients. Perhaps of even greater potential medical importance is the proposed role of chronic HCMV infections in diseases such as coronary atherosclerosis and human cancers, two areas of active research (Everett *et al.*, 1992; Zhou *et al.*, 1996; Hosenpud, 1999; Zhu *et al.*, 1999; Streblow *et al.*, 2000).

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45

HCMV persistence in the population: potential transplacental transmission

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Congenital cytomegalovirus infection and the placenta

Congenital CMV infection

Human cytomegalovirus (CMV) is a ubiquitous virus that causes asymptomatic infections in healthy individuals (for review see Pass, 2001). Because breast feeding (Stagno *et al.*, 1980), exposure to young children (Pass *et al.*, 1987) and sexual contact (Fowler and Pass, 1991) are major risk factors for infection, most adults are seropositive. Diverse organs and specialized cells, including polarized epithelial cells (Tugizov *et al.*, 1996) and endothelial cells (Fish *et al.*, 1998; Maidji *et al.*, 2002), are susceptible to CMV infection. CMV establishes latent infection in granulocyte-macrophage progenitors (Kondo *et al.*, 1996) and reactivates upon cellular differentiation (Hahn *et al.*, 1998; Soderberg-Naucler *et al.*, 1997). Congenital CMV infection is estimated to affect 1 to 3% of infants in the United States annually and remains an important public health problem causing significant morbidity and mortality (for review see Britt, 1999).

It has long been appreciated that maternal neutralizing antibodies reduce the risk of symptomatic congenital disease in the fetus (Ahlfors *et al.*, 1984; Boppana and Britt, 1995; Fowler *et al.*, 2003; Stagno *et al.*, 1982). The importance of adaptive immunity to CMV is apparent in women with primary infection, often with low-avidity neutralizing antibodies (Boppana and Britt, 1995; Lazzarotto *et al.*, 1998; Revello *et al.*, 2002). Approximately 15% of these women spontaneously abort in early gestation (Griffiths and Baboonian, 1984). Examination of placentas infected with CMV in vitro and in utero has suggested potential routes of virus transmission from the uterus to the placenta (Fisher *et al.*, 2000; Pereira *et al.*, 2003). Importantly, these studies suggest that placental involvement precedes virus transmission and infection of the embryo/fetus. Progression of infection hinges on maternal immunity to CMV, the mechanics of cytotrophoblast development and the presence of other pathogens at the maternal–fetal interface. In this chapter, we describe patterns of CMV infection in early gestation, routes of viral transmission at the maternal-fetal interface and dysregulation of cytotrophoblast differentiation and function secondary to CMV infection in vitro.

CMV infects specialized cells in the placenta

Numerous reports indicate that placentas from pregnancies complicated by congenital CMV infection contain viral DNA and proteins (Benirschke and Kaufmann, 2000; Muhlemann *et al.*, 1992; Nakamura *et al.*, 1994; Sinzger *et al.*, 1993). Later in pregnancy, CMV infection is associated with premature delivery and, in 25% of affected infants, intrauterine growth retardation (Istas *et al.*, 1995), outcomes that are often associated with placental pathologies. CMV replicates in cytotrophoblasts isolated from early and late gestation placentas in vitro (Fisher *et al.*, 2000; Halwachs-Baumann *et al.*, 1998; Hemmings *et al.*, 1998). The routes of virus transmission and the types of immune responses elicited are likely linked to the unusual nature of cytotrophoblast interactions with maternal cells at the uterine-placental interface. A diagram of the maternal-fetal interface midway through gestation with potential sites of CMV infection is shown in Fig. 45.1.

Placental development in early gestation

Diverse cell types in the uterus

Immunologically competent cells are detected in the uterine endometrium and decidua (Kamat and Isaacson,

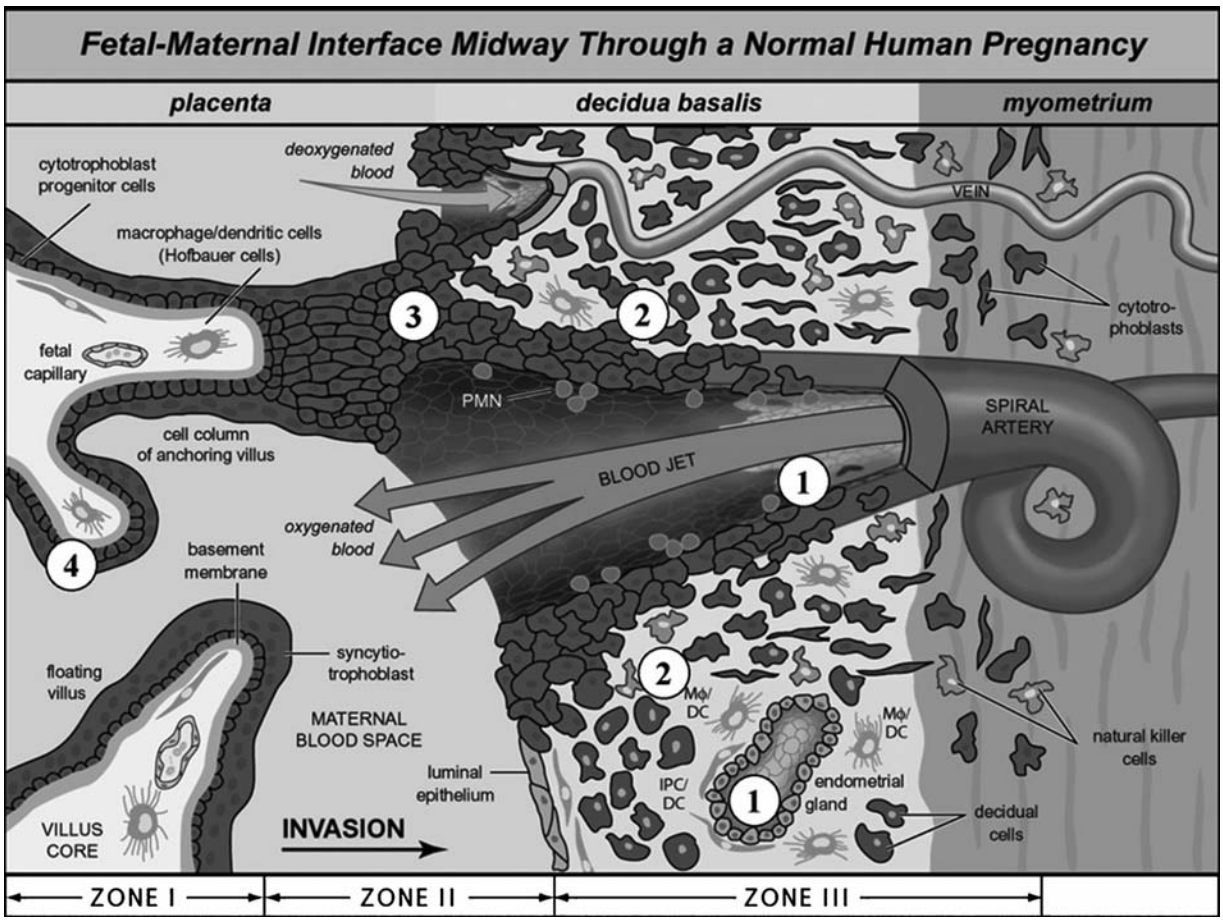

Fig. 45.1. Diagram of the histologic organization of the human maternal-fetal interface at midgestation. In this location, cytotrophoblasts, which are specialized (fetal) epithelial cells of the placenta, differentiate and invade the uterine wall, where they also breach maternal blood vessels. The basic structural unit of the placenta is the chorionic villus, composed of a stromal core with blood vessels, surrounded by a basement membrane and overlain by cytotrophoblast progenitor cells. As part of their differentiation program, these cells detach from the basement membrane and adopt one of two lineage fates. They either fuse to form the syncytiotrophoblasts that cover floating villi or join a column of extravillous cytotrophoblasts at the tips of anchoring villi. The syncytial covering of floating villi mediates nutrient, gas, and waste exchange and transfer of IgG from maternal blood to the fetus (Zone 1). The anchoring villi, through the attachment of cytotrophoblast columns, establish physical connections between the fetus and the mother (Zone II). Invasive cytotrophoblasts penetrate the uterine wall up to the first third of the myometrium (Zone III). A portion of the extravillous cytotrophoblasts home to uterine spiral arterioles and remodel these vessels by destroying their muscular walls and replacing their endothelial linings. To a lesser extent they also remodel uterine veins. At term, few cytotrophoblast progenitor cells remain, the syncytiotrophoblast layer thins, and the volume of the stromal cores expand. Sites proposed as routes of CMV infection in utero are numbered 1 to 4. Diagram modified from (Hoang *et al.*, 2001).

1987). Early studies of leukocyte antigens by using immunohistologic approaches suggested that cells in the endometrium of cycling women in the mid-secretory phase (7–10 days after ovulation) resemble leukocytes in early gestation decidua (King *et al.*, 1989). Granulated lymphocytes with an unusual antigenic phenotype (CD56+ high, CD16–), known as natural killer (NK) cells, constitute a substantial proportion of these cells (Bulmer *et al.*, 1991; Starkey *et al.*, 1988), increasing from the proliferative endometrium to the late secretory endometrium. Macrophages increase prior to menstruation (Kamat and Isaacson, 1987). CD83+ dendritic cells in the uterine stratum basale are present in the non-pregnant and pregnant uterus (Kammerer *et al.*, 2000; Soilleux *et al.*, 2002).

In response to implantation, the uterine lining develops into the decidua, which is maintained by progesterone

(Norwitz *et al.*, 2001). Interglandular tissues increase in quantity, and the cytoplasm of resident stromal cells is distended with glycogen, lipid and vimentin-type intermediate filaments (Fig. 45.1, Zone III). Temporal and spatial expression of growth factors and cytokines (e.g., insulin-like growth factor 1 and its binding protein) suggests that these molecules may influence decidualization (Crossey *et al.*, 2002). Decidual granular leukocytes intermingle with resident maternal cells and invasive fetal cells (Zone III) (Drake *et al.*, 2001; Kamat and Isaacson, 1987; Red-Horse *et al.*, 2001; Starkey *et al.*, 1988). These immune cells are involved in innate pattern recognition, mostly NK cells with some macrophages, dendritic cells and T-lymphocytes. Novel patterns of cytokine/chemokine expression in the decidua, as well as specialized adhesion molecules on uterine vessels (Kruse *et al.*, 1999), probably attract this unusual leukocyte population, which functions in immunity and cytotrophoblast differentiation. Dendritic cell protein ICAM-3-grabbing non-integrin (DC-SIGN+) cells, occasionally found in the endometrium, are abundant in the decidua and associate with NK cells (Kammerer *et al.*, 2003, 1999; Pereira *et al.*, 2003; Soilleux *et al.*, 2002). The unusual immune cell population in the decidua suggests that when macrophage/dendritic cell progenitors (MΦ/DC) latently infected with CMV are attracted to the endometrium and early gestation decidua, CMV could be reactivated in the presence of inflammatory stimuli.

Development of the hemochorial human placenta

The embryo's acquisition of a supply of maternal blood is a critical hurdle in pregnancy maintenance. The mechanics of this process are accomplished by the placenta's specialized epithelial cells, termed cytotrophoblasts. The histology of the maternal-fetal interface is diagrammed in Fig. 45.1. The placenta is composed of individual units termed chorionic villi, each with a connective core that contains fetal blood vessels and numerous macrophages (Hofbauer cells) that often lie under a thick basement membrane (Fig. 45.1, Zone I). Placentation is a stepwise process whereby cytotrophoblast progenitor cells, attached to the basement membrane as a polarized epithelium, leave the membrane to differentiate along one of two independent pathways depending on their location. In floating villi, they fuse to form a multinucleate syncytial covering attached at one end to the tree-like fetal portion of the placenta (Zone I). The rest of the villus floats in a stream of maternal blood, which optimizes exchange of substances between the mother and fetus across the placenta. In the pathway that gives rise to anchoring villi, which attach the placenta to the uterine wall (Zone II), cytotrophoblasts aggregate into cell columns of non-polarized mononuclear cells that attach to and then penetrate the uterine wall. The ends of the columns terminate within the superficial endometrium, where they give rise to invasive cytotrophoblasts. During interstitial invasion a subset of these cells, either individually or in small clusters, comingles with resident decidual, myometrial and immune cells. During endovascular invasion, masses of cytotrophoblasts open the termini of uterine arteries and veins they encounter, then migrate into the vessels, thereby diverting maternal blood flow to the placenta (Zone III). In arterioles, cytotrophoblasts replace the endothelial lining and partially disrupt the muscular wall, whereas in veins, they are confined to the portions of the vessels near the inner surface of the uterus. Together, the two components of cytotrophoblast invasion anchor the placenta to the uterus and permit a steady increase in the supply of maternal blood that is delivered to the developing fetus.

Invasive cytotrophoblasts modulate the expression of stage-specific antigens

During uterine remodeling, cytotrophoblasts switch from an epithelial to a mesenchymal type (Table 45.1). Cytotrophoblasts express novel adhesion molecules and proteinases that enable the cells' attachment and invasion, as well as immune modulating factors that play a role in maternal tolerance of the hemiallogeneic fetus (Cross *et al.*, 1994; Norwitz *et al.*, 2001). Interstitial invasion requires downregulation of integrins characteristic of epithelial cells ($\alpha6\beta4$) and novel expression of $\alpha1\beta1$, $\alpha5\beta1$ and $\alpha V\beta3$ (Damsky *et al.*, 1994). Endovascular cytotrophoblasts that remodel maternal blood vessels transform their adhesion receptor phenotype to resemble the endothelial cells they replace (Fig. 45.1, site 1) turning on the expression of VE-(endothelial) cadherin, platelet-endothelial adhesion molecule-1 and vascular endothelial adhesion molecule-1 (Damsky and Fisher, 1998; Zhou *et al.*, 1997).

Degradation of the basement membrane and extracellular matrix of the uterine stroma is precisely regulated during placentation (Fisher *et al.*, 1985). Cytotrophoblasts upregulate urokinase-type plasminogen activator (uPA) (Queenan *et al.*, 1987; Solberg *et al.*, 2003), matrix metalloproteinase-9 (MMP-9) (Librach *et al.*, 1991) and inhibitors such as tissue inhibitor of metalloproteinases-3, a likely regulator of proteolytic activity and invasion depth (Bass *et al.*, 1997) (Table 45.1). Molecules that may function in maternal immune tolerance are also produced, such as the non-classical major histocompatibility complex (MHC) class Ib molecule HLA-G (Kovats *et al.*, 1990; McMaster *et al.*, 1995) and interleukin-10 (IL-10) (Roth *et al.*, 1996; Roth and Fisher, 1999). This remarkable transformation, evidenced by novel expression of differentiation molecules

and invasiveness, underscores the extraordinary plasticity of cytotrophoblasts.

CMV infects the placenta in vitro and in utero

Potential routes for CMV transmission

The cellular organization of the placenta suggests potential routes by which CMV infection spreads from the uterus, first to the placenta and then to the embryo/fetus (Fig. 45.1) (Fisher *et al.*, 2000; Pereira *et al.*, 2003). One likely site of transmission is within the uterine wall (sites 1 and 2). Interstitial invasive cytotrophoblasts could encounter infected endometrial glands, uterine blood vessels and decidual granular leukocytes. Endovascular cytotrophoblasts could encounter infected endothelial and vascular smooth muscle cells, as well as maternal immune cells. Once cytotrophoblasts within the uterine wall become infected (site 2), CMV might spread in a retrograde manner through the cell columns to the anchoring chorionic villi (site 3). In the villus stromal cores, virus could be transmitted from infected cytotrophoblasts to fibroblasts, fetal macrophages and possibly endothelial cells that line chorionic vessels. This conjecture is based on focal patterns of CMV protein expression in the placenta (Fisher *et al.*, 2000; Muhlemann *et al.*, 1992). Infected MΦ/DC and sloughed endothelial cells seem likely candidates for entering the venous circulation of the placenta and subsequently carrying the infection via the placental circulation to the fetus. Another likely site of transmission is across the syncytiotrophoblast layer that covers floating chorionic villi (site 4). These placental cells, in direct contact with maternal blood, express the neonatal Fc receptor (FcRn), a molecule that facilitates maternal IgG transfer and passive immunization of the fetus (Simister *et al.*, 1996). The syncytium may allow passage of CMV virions complexed with maternal IgG to the underlying layer of cytotrophoblast progenitor cells that could become infected in the presence of virus-binding antibodies with low avidity (Boppana and Britt, 1995; Fisher *et al.*, 2000; Lazzarotto *et al.*, 1997; Pereira *et al.*, 2003; Revello *et al.*, 2002). Accordingly, adaptive and innate immune responses that reduce infectious virions at the uterine–placental interface likely play a central role in preventing transmission.

CMV protein expression in placental cells in chorionic villi infected in vitro and in utero

Clues about potential routes of prenatal CMV infection emerged from a model tissue culture system (Fig. 45.2(a)). Chorionic villi are plated on filters coated with Matrigel, an extracellular matrix, infected with virus and then cultured from 2 to 4 days. Experiments that used this model revealed an unexpected pattern of CMV infection (Fisher *et al.*, 2000). Briefly, tissue sections of villus explants that were infected for several days were double-stained with anti-cytokeratin to identify trophoblast cells and with a monoclonal antibody to CMV immediate-early (IE) proteins to identify infected cells. Notably, syncytiotrophoblasts that cover the villus surface were not infected and failed to stain for CMV IE proteins, whereas nuclear staining of small, isolated clusters of underlying cytotrophoblast progenitor cells was observed (Fig. 45.1, site 4). In some tissues, CMV IE protein expression was also detected in cytotrophoblasts

Table 45.1. Selected differentiation molecules expressed by placental trophoblasts[a]

	Floating villus		Column CTB		Invasive CTB	
	CTB	STB	Proximal	Distal	Interstitial	Endovascular
	zone I		Zone II		Zone III	
Receptors (integrins)						
α1[b]	−	−	−	−	+	+
α4[b]	+	−	+	+	+	+
αV[c]	+	−	+	+	+	+
β1[b, d]	−	−	+	+	+	+
β3[c]	−	−	−	+	+	+
β4[b]	+	−	+	+/−	−	−
β5[c]	+	−	+	−	−	−
β6[c, e]	+	−	+	−	−	−
Proteinase and inhibitors						
MMP-9[f]	−	−	+	+	+	+
TIMP-3[f]	−	−	+	+	+	+
uPA[f]	−	−	+	+	+	+
Immune molecules						
HLA-G[g]	−	−	−	+	+	+
IL-10[h]	+	+	+	+	+	−
FcRn[i]	−	+	−	−	−	−

[a]Adapted from (Damsky and Fisher, 1998) and (Zhou *et al.*, 1997) with permission. Abbreviations: CTB, cytotrophoblasts; STB, syncytiotrophoblasts; MMP, metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; uPA, urokinase-type plasminogen activator; IL, interleukin; FcRn, neonatal Fc receptor; –, not expressed; +, expressed.
[b]Published results:(Damsky *et al.*, 1992; Zhou *et al.*, 1993).
[c](Zhou *et al.*, 1997, Maidji *et al.*, 2006).
[d]Detected in second-trimester villus.
[e]Detected at site of column formation in second trimester.
[f]Published results: (Librach *et al.*, 1991).
[g](McMaster *et al.*, 1995).
[h](Roth and Fisher, 1999).
[i](Simister *et al.*, 1996).

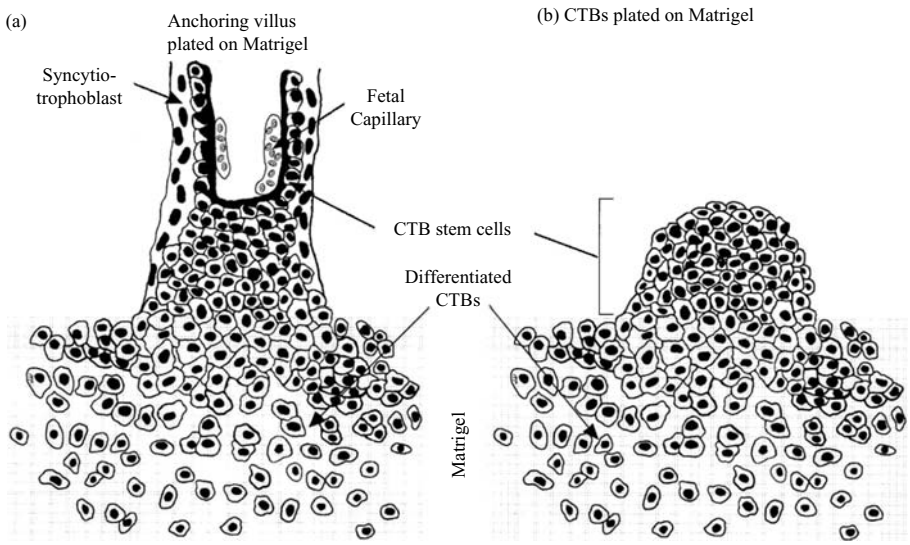

Fig. 45.2. Culture models for studying CMV infection of placental explants and cells. (a) Diagram of an anchoring villus explant attached to a Matrigel substrate via cytotrophoblasts (CTBs) that migrate from the cell columns. (b) Diagram of purified differentiating cytotrophoblasts cultured on Matrigel. The cytotrophoblast progenitor cells aggregate, invade the matrix, and express stage-specific molecules (see Table 45.1). For infection, CMV is added to the medium bathing the explants and purified cytotrophoblasts.

in the cell columns of anchoring villi (Fig. 45.1, site 3). The staining patterns observed when placentas were infected in utero had remarkable similarities to and differences from those observed after infection in vitro. Sometimes, patterns of CMV-infected cells were virtually indistinguishable from those found after infection in vitro; in some locations, isolated clusters of cytotrophoblasts underlying the syncytium were the only cells infected (Fig. 45.3(b)). At other times, nearly all the cytotrophoblast progenitor cells, in highly infected tissues, expressed CMV IE proteins (Fig. 45.3(c), (d)). Comparatively fewer syncytial nuclei stained, but numerous cells within the villus cores stained for viral proteins, including fibroblasts, endothelial cells and macrophages. These studies suggested that in vitro infection is a model for the initial steps in placental infection, whereas in utero infection shows virus transmission from trophoblasts to other cell types in the villus core. The interplay between pathogens and immune responses in other tissues suggest that CMV infection might often occur in the context of the microbial ecology of female reproductive tissues.

Pathogenic microorganisms at the placental–decidual interface

In a study using PCR-based strategies, the presence of viral and bacterial DNA was assessed in biopsy specimens of the decidua and adjacent placentas of 282 healthy pregnancies (McDonagh *et al.*; Pereira *et al.*, 2003). Overall, CMV DNA was detected in 69% of specimens, and CMV with bacteria was detected in 38%. When found in isolation, CMV was detected in 27% of placental samples. Other pathogens included herpes simplex virus type 1 (HSV-1) in 3%; HSV-2 in 9%, and more than one bacterium in 15%. Sixteen percent of placental samples were negative for these pathogens. These findings suggest that early gestation placentas frequently contain DNA from viral and bacterial pathogens.

Detailed analysis of paired first-trimester decidual and placental biopsy specimens from individual pregnancies showed that some pathogens were present in both. CMV DNA was detected in 89% of the decidual samples and 63% of the placentas. When CMV was found in isolation in the decidua (40%), virus was also sometimes present in the placenta (26%). In contrast, when bacterial DNA was detected in the placenta (11%), signals were less frequently found in the decidual samples (6%). Together these results suggest that CMV can be selectively transferred from the decidua, a potential reservoir, to the adjacent placenta. When the effects of gestational age were examined, CMV DNA, with or without other pathogens, was detected in 63% of first-trimester placentas and 74% of second-trimester placentas. Together, samples with both CMV and bacterial DNA increased from 31% in the first trimester to 44% in the second trimester, whereas CMV alone was reduced in the second trimester. Fewer second-trimester placentas were negative for all pathogens. These studies suggested that (a) CMV is commonly present at the uterine-placental

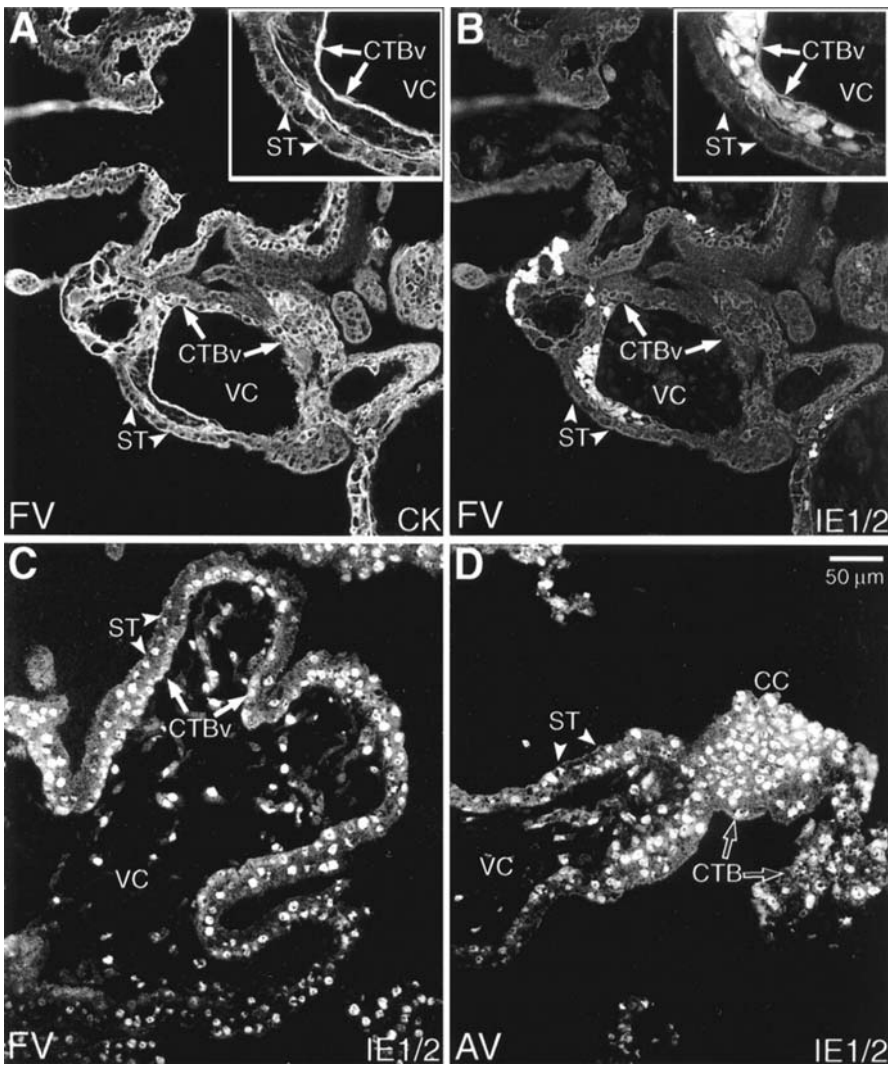

Fig. 45.3. Natural infection of chorionic villi with CMV in utero in cytotrophoblasts and other cells. Both floating villi (FV) (a–c) and anchoring villi (AV) (d) are shown. Tissues were analyzed by using immunolocalization techniques for expression of (a) cytokeratin (CK) and (b, d) CMV IE proteins. (b) In some floating villi, clusters of CMV-infected villous cytotrophoblast progenitors (CTBv) (arrows) underlying the syncytium (ST) (arrowheads) were the only sites of antibody reactivity. Sometimes, numerous cells throughout the villi stained with anti-CMV antibody. (c) Nuclei of syncytiotrophoblasts, villous cytotrophoblasts and stromal components expressed CMV proteins. (d) The same pattern of immunoreactivity was seen in infected anchoring villi. Additionally, cytotrophoblasts in cell columns (CC) stained brightly. VC, villus core.

interface together with pathogenic bacteria, (b) infection tends to increase in the second trimester, and (c) virus is selectively transmitted to the adjacent placenta.

Neutralizing antibodies to CMV gB in placental syncytiotrophoblasts

Development of neutralizing antibodies is delayed when primary CMV infection occurs shortly before or during gestation (Boppana and Britt, 1995; Lazzarotto *et al.*, 1997; Revello *et al.*, 2002), whereas high titers indicate resolution of acute infection and/or reactivation. Antibody responses to CMV in the group of donors from whom paired biopsy specimens were obtained showed that, with one exception, the donors were seropositive with a range of neutralizing activity. Briefly, neutralizing activity in IgG purified from the conditioned medium of biopsy specimens was evaluated. Ten women had low neutralizing titers (0% to 32%), nine had moderate titers (43% to 67%) and four had high titers (70 to 98%).

Some serologic evidence suggests that reinfection with new CMV strains in seropositive women might be associated with symptomatic fetal infection (Boppana *et al.*, 1999). To determine whether multiple strains colonize the placental-decidual interface, a region of the gB gene with characteristic nucleotide differences was sequenced. Sequence analysis of a small number of CMV-positive samples revealed that the gB genotypes were similar to variants in groups 1, 2 and group 3 (Chou and Dennison, 1991). Paired decidua and adjacent placenta from a seropositive donor without detectable neutralizing antibodies contained a mixture of gB genotypes, suggesting that different CMV strains could infect the maternal-fetal interface early in the course of maternal infection.

Patterns of CMV-infected-cell proteins in the decidua and placenta

Decidual biopsy specimens that contained CMV DNA were studied by immunofluorescence confocal microscopy to determine whether viral proteins could be detected (McDonagh *et al.*, 2004; Pereira *et al.*, 2003). Tissue sections of decidual biopsy samples were incubated with a pool of monoclonal antibodies to CMV-infected-cell proteins and to gB, an abundant virion envelope glycoprotein. Staining revealed islands of infected resident uterine and fetal cells, as well as innate immune cells among much larger uninfected areas. Several common staining patterns emerged. In the most highly affected samples, CMV-infected-cell proteins were found in the nuclei and cytoplasm of glandular epithelium (Fig. 45.4(a), *a–c*), vascular endothelium and endovascular cytotrophoblasts (Fig. 45.4(a), *d–f*). Resident decidual cells positive for insulin growth factor binding protein 1 (IGFBP-1) also stained brightly (Fig. 45.4(a), *g–l*). These data indicate that CMV infects a diverse population of maternal cells within the uterine wall and fetal invasive cytotrophoblasts. Innate immune cells showed a staining pattern that was distinctly different from that of CMV-infected cells, suggesting phagocytosis of enveloped virions. Macrophages (CD68+) contained cytoplasmic vesicles that stained strongly for CMV gB (Fig. 45.4(b), *a–c*). Some gB-positive cells also stained for DC-SIGN (Kammerer *et al.*, 2003; Soilleux *et al.*, 2001) (Fig. 45.4(b), *d–f*). NK (CD56+) cells were often dispersed among Mφ/DC that were filled with gB-positive vesicles (Fig. 45.4(b), *g*). Occasionally, striking numbers of NK cells and Mφ/DC intermingled (Fig. 45.4(b), *h* and *i*). Additionally, neutrophils inside uterine blood vessels were found near endothelial cells and decidual cells that expressed CMV-infected-cell proteins, suggesting phagocytosis (Fig. 45.4b, *j–l*). These observations suggested that the uterus serves as a reservoir for CMV virions that could potentially infect the placenta.

Different patterns of CMV infection in the decidua mirrored in the adjacent placenta

Examination of CMV proteins in paired decidual and placental biopsy specimens showed three staining patterns (Pereira *et al.*, 2003). In the first, islands in both decidual and placental compartments stained strongly for CMV-infected-cell proteins. This pattern predominated in samples from donors with low neutralizing titers and a few with intermediate titers and other pathogens. In the decidua, cytokeratin-positive glandular epithelial cells, endovascular cytotrophoblasts in remodeled uterine blood vessels, and interstitial cytotrophoblasts were sometimes positive. Resident decidual cells strongly stained for viral proteins, suggesting that these cells were permissive for viral replication. In the adjacent portions of the placenta, floating villi contained syncytiotrophoblasts and cytotrophoblast progenitor cells expressing CMV-infected-cell proteins that localized to the nuclei and cytoplasm. Abundant vesicles amassed close to the plasma membrane of the villus surface and contained gB. In regions with infected syncytiotrophoblasts, fibroblasts and fetal capillaries in the villus core expressed infected-cell proteins. Invasive cytotrophoblasts in developing cell columns that anchor the placenta to the uterine wall also stained. In contrast, Mφ/DCs within the villus stromal cores contained infected-cell proteins in cytoplasmic vesicles but not in the nuclei, suggesting phagocytosis.

In the second group of paired biopsy specimens, the number of cells that stained for CMV-infected-cell proteins was reduced in the decidua, and occasional focal infection was found in the placenta. This pattern predominated in samples from donors with low to intermediate neutralizing titers, several of which contained other pathogens. In the decidua, CMV replication was detected in some glandular epithelial cells and decidual cells. In the interstitium, Mφ/DCs were abundant throughout, especially near infected glands and blood vessels. These cells contained gB-positive cytoplasmic vesicles but were not infected. Sometimes the adjacent placentas contained small clusters of cytotrophoblast progenitor cells that expressed CMV-infected-cell proteins. Isolated gB-containing vesicles were present in the overlying syncytiotrophoblast layer. In the villus core, Mφ/DCs containing CMV gB-positive vesicles were often observed. In other placental biopsies, only gB-containing vesicles were detected in syncytiotrophoblasts and villus core Mφ/DCs without infection.

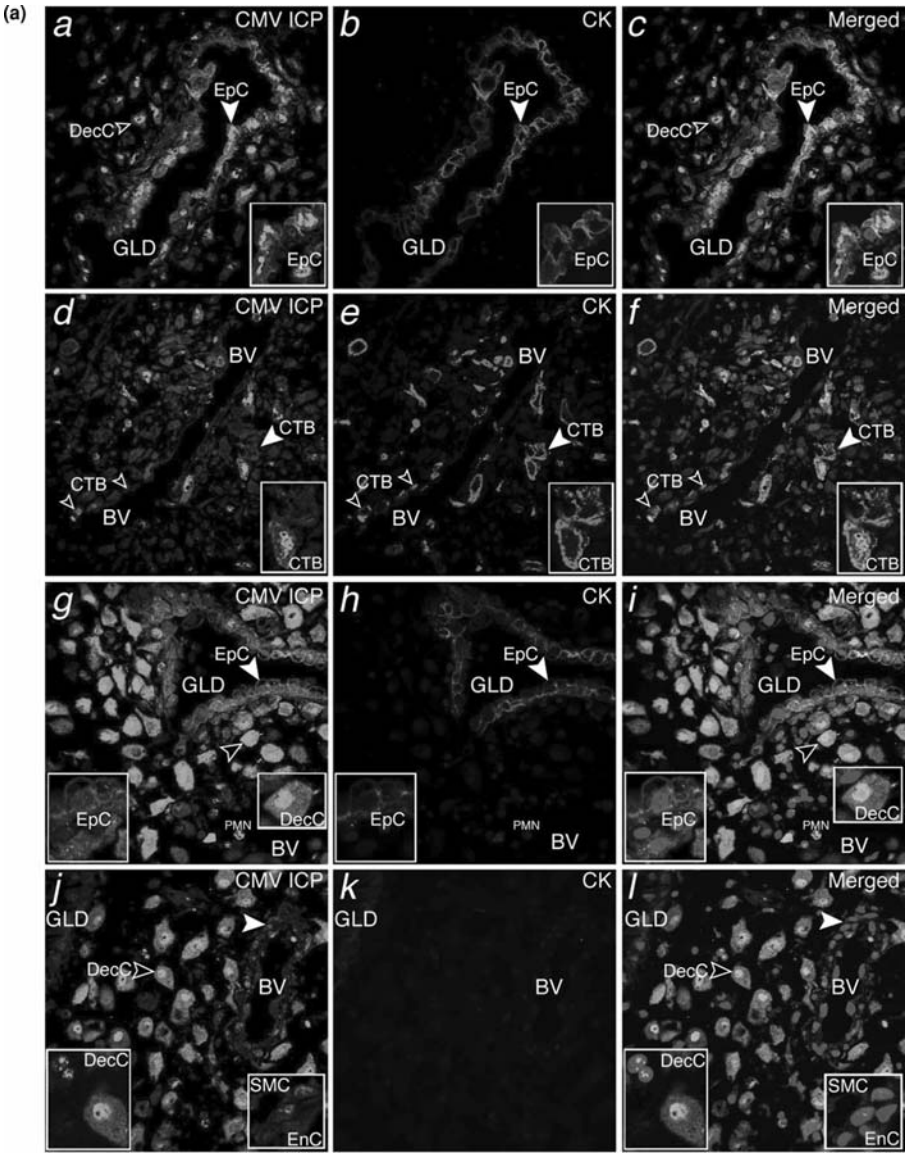

Fig. 45.4. Panel (a): CMV replicates in diverse cell types in uterine decidua. CMV infects endometrial glands (GLD), uterine blood vessels (BV), resident decidual cells (DecC) and cytotrophoblasts (CTB) in the decidua. (*a*)–(*c*), Decidual biopsy specimens stained for CMV-infected-cell proteins (ICP, green) and cytokeratin (CK, red), which identified epithelial cells (EpC). (*d*)–(*i*), CMV-infected interstitial and endovascular CTB and DecC. (*j*)–(*l*), Endothelial cells (EnC) and smooth muscle cells (SMC) of uterine blood vessels (BV) are infected. Panel (b): Abundant innate immune cells infiltrating the decidua contain CMV proteins. (*a*)–(*c*) CMV gB (green), macrophages (Mφ/DC, CD68, red). (g)–(h) DC-SIGN+ (green) macrophage/dendritic cells (Mφ/DC) take up CMV gB (red). (*g* (and) *h*) CD56+ (green) natural killer (NK) cells target infection sites. (*i*) DC-SIGN+ cells containing gB. (*j*)–(*l*) Neutrophils (PMN) with phagocytosed proteins from virus-infected cells and endothelial cells (EnC) positive for von Willebrand factor (vWF) in blood vessels (BV). "Merged" indicates colocalized proteins (yellow). Large arrowheads indicate area shown in insets. (See color plate section.)

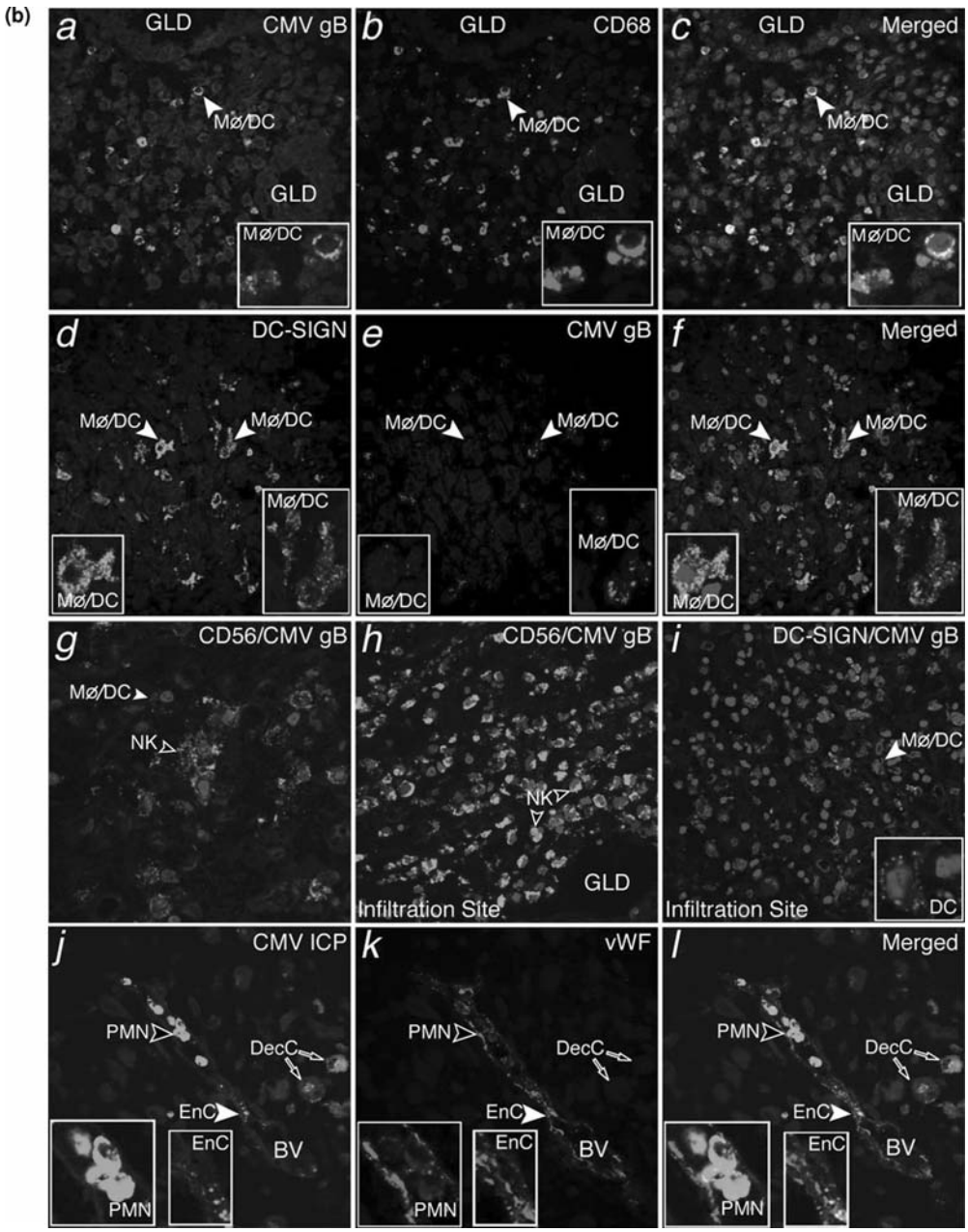

Fig. 45.4. (*cont.*) (See color plate section.)

In the last group of paired biopsy specimens, few cells stained for CMV-infected-cell proteins in the decidua, and none were found in the placenta. This pattern predominated in samples from donors with intermediate to high neutralizing titers, several of which contained other pathogens.

In the decidua, neutrophils with viral proteins were found in uterine blood vessels near infected cells. In the adjacent portions of the placenta, syncytiotrophoblasts contained numerous CMV gB-positive vesicles but were not infected. In villus core Mφ/DCs, gB accumulated in large cytoplasmic vesicles. When placentas were stained for IgG, syncytiotrophoblasts contained many positive vesicles, and gB colocalized with a small subset of them. In villus core Mφ/DCs, some gB-staining vesicles colocalized with the more abundant IgG-positive

vesicles. FcRn-positive vesicles at the apical and basolateral membranes suggested IgG transcytosis in syncytiotrophoblasts (Simister and Story, 1997). In some cases, the presence of viral nucleocapsids in syncytiotrophoblasts was confirmed by electron microscopy.

These studies concluded that CMV is commonly present at the maternal-fetal interface, one possible explanation for why pregnant women shed virus from the cervix (Collier *et al.*, 1995; Shen *et al.*, 1993; Stagno *et al.*, 1975). Bacteria were often found in donors with intermediate to high neutralizing titers whose uninfected placentas contained virion proteins suggesting limited CMV replication in the decidua. Reactivation from decidual MΦ/DCs might occur as a consequence of inflammatory responses to pathogenic bacteria and could depend on the number of latently infected MΦ/DCs infiltrating the uterus (Cook *et al.*, 2002; Hahn *et al.*, 1998; Soderberg-Naucler *et al.*, 2001). Placentas from healthy pregnant donors contained isolated areas of infection that were a small part of the whole tissue. Since these tissues were from normal pregnancies, placental infection that leads to fetal transmission likely involves the decidual and placental components that stained for infected-cell proteins, i.e., an exacerbation of the situation found in samples from women with the lowest neutralizing titers and some with intermediate titers as well as bacterial pathogens.

Coordinated immune responses suppressed CMV infection of the placenta in women with intermediate to high neutralizing titers, one explanation for a correlation between high-avidity IgG and protection against vertical transmission (Boppana and Britt, 1995; Revello *et al.*, 2002). The most remarkable result is that women with uncomplicated pregnancies had suppressed infection in the decidua. Virion-IgG complexes may be transported to the placenta without infection, a process that demonstrates the efficacy of innate and adaptive immunity. CMV infection of the decidua is a novel paradigm and further illustrates how this virus utilizes host immunity (Mocarski, 2002) by exploiting maternal hyporesponsiveness.

Analysis of CMV DNA and proteins expressed in placentas from uncomplicated deliveries by PCR and immunohistochemistry showed evidence of transplacental transmission (McDonagh *et al.*, 2006). CMV DNA was detected in 62% of term placentas examined. In biopsy specimens from placentas with high levels of CMV DNA and low maternal neutralizing titers, fetal blood vessels contained leukocytes with viral replication proteins. Some cord blood samples contained CMV DNA, confirming viral replication. In placentas with low levels of viral DNA and high neutralizing titers, villus core macrophages and dendritic cells contained CMV gB, comparable to infection in early gestation, suggesting virion uptake without transmission. Together the results showed that CMV infection spreads from villus cytotrophoblasts to stromal fibroblasts, placental blood vessels and fetal leukocytes in late gestation. Over 5% of uncomplicated deliveries contained CMV replication proteins, suggesting a higher incidence of transplacental transmission and asymptomatic congenital infection than previously thought.

Complexes of IgG and CMV virions transcytosed from maternal circulation across syncytiotrophoblasts to underlying cytotrophoblasts

Immunohistochemical analysis of early gestation biopsy specimens showed an unusual pattern of CMV replication proteins in underlying cytotrophoblast progenitor cells. Whereas syncytiotrophoblasts were spared in placentas with low to moderate CMV neutralizing titers, cytotrophoblasts were infected, suggesting virion transcytosis from maternal blood (Pereira *et al.*, 2003). Early steps of CMV infection and the role of FcRn were examined using the villus explant model (Fig. 45.2(a)) and polarized epithelial cells (Tugizov *et al.*, 1996). The results showed that (i) maternal IgG modulates CMV infection in chorionic villi, (ii) FcRn transcytoses IgG–virion complexes that retain infectivity with low neutralizing antibodies, (iii) villus core macrophages capture transcytosed IgG-virions, (iv) IgG and gB accumulate in caveolae and (v) CMV DNA is present in syncytiotrophoblasts without viral replication. Receptor-mediated transport and caveolar endocytosis explain the infection patterns in villus cytotrophoblasts and CMV virion gB accumulation in vesicular compartments in utero (Pereira *et al.*, 2003). The rapid kinetics of receptor-mediated transport of IgG–virions was similar in syncytiotrophoblasts and polarized T-84 intestinal epithelial cells expressing FcRn: transcytosed immune complexes were detected in villus core macrophages (explants), underlying cytotrophoblasts or the basal medium (cells). In villus explants, IgG–virion transcytosis and macrophage uptake were blocked with trypsin treatment and soluble protein A. The results suggest that CMV virions could disseminate to the placenta by co-opting the receptor-mediated transport pathway for IgG. It was recently reported that passive immunization with hyperimmune IgG at midgestation prevents congenital disease and growth restriction in infected infants of mothers with primary CMV infection (Nigro *et al.*, 2005). This remarkable outcome suggests once CMV replication has been interrupted, functional villi develop that transport neutralizing IgG to the fetus, reducing dissemination.

Receptors for CMV virions are developmentally regulated in cytotrophoblasts

CMV replication in distinct cytotrophoblast populations suggests that virion receptors could be developmentally regulated as these specialized cells proceed along the differentiation pathway from the fetal to the maternal compartment (Fig. 45.1). Function-blocking methods and immunohistochemical analysis were used to correlate infection with expression of CMV receptors in cytotrophoblasts in situ and in vitro (Maidji *et al.*, 2006). In placental villi, syncytiotrophoblasts express a virion receptor, epidermal growth factor receptor (EGFR) (Wang *et al.*, 2003), but lack integrin coreceptors, and endocytosis occurs without replication. IgG–CMV virion complexes transcytosed by FcRn reach underlying cytotrophoblasts (Maidji *et al.*, 2006). Some EGFR-expressing cells selectively initiate expression of a coreceptor, αV integrin (Feire *et al.*, 2004; Wang *et al.*, 2005), and focal infection can occur. In cell columns, proximal cytotrophoblasts lack receptors, and distal cells express integrins α1β1 and αVβ3 but remain uninfected. In the uterine decidua, invasive cytotrophoblasts expressing integrin coreceptors upregulate EGFR, thereby dramatically increasing susceptibility. These findings indicate that virion engagement with receptors in the placenta (i) changes as cytotrophoblasts differentiate and (ii) correlates with spatially distinct sites of CMV replication in maternal and fetal compartments in utero.

CMV infection dysregulates cytotrophoblast differentiation/invasion in vitro

CMV replicates in placental cytotrophoblasts in vitro

Several groups have reported that cytotrophoblasts isolated from early gestation (Fisher *et al.*, 2000; Hemmings *et al.*, 1998) and term placentas (Halwachs-Baumann *et al.*, 1998) are susceptible to CMV infection. A detailed examination of the viral life cycle was done using an in vitro model of progenitor cytotrophoblasts from chorionic villi plated as a monolayer on Matrigel and cultured from 2 to 4 days after infection (see Fig. 45.2(b)) (Fisher *et al.*, 2000; Librach *et al.*, 1991). Under these conditions the cells form aggregates, analogous to cell columns, and differentiate along the invasive pathway. In CMV-infected cells, nuclear staining for IE proteins was detected by 24 hours and cytoplasmic staining for gB was detected by 72 hours in 20 to 40% of the cells. There was an increase in the titers of intracellular and progeny virions released during the culture period, establishing that differentiating cytotrophoblasts are fully permissive for CMV replication.

Investigation of the effects of viral infection in cytotrophoblasts showed considerable changes, evidenced by dysregulated expression of stage-specific adhesion and immune molecules, as well as metalloproteinases and their inhibitors (Fisher *et al.*, 2000; Maidji *et al.*, 2002; Yamamoto-Tabata *et al.*, 2004). Importantly, the cells' central function, invasion, was significantly impaired following infection.

CMV infection in vitro downregulates cytotrophoblast expression of HLA-G

In healthy placentas, the non-classical MHC class Ib molecule HLA-G is expressed in differentiating cytotrophoblasts, particularly cells in anchoring villi with an increasing gradient of expression in the distal columns that is maintained once the cells enter the uterine wall (Kovats *et al.*, 1990; McMaster *et al.*, 1995). Immunolocalization experiments showed that CMV infection of differentiating cytotrophoblasts in vitro downregulates expression of HLA-G (Fisher *et al.*, 2000). At late times when high levels of CMV gB were detected (Fig. 45.5(a)), staining for HLA-G was either greatly reduced or lost (Fig. 45.5(b)). This was in contrast to cells that were not infected with CMV and stained with anti-HLA-G.

Several CMV genes downregulate expression of classical MHC class Ia molecules (for review see Ploegh, 1998). Studied in the context of cytotrophoblasts infected with CMV mutants in which all of the genes known to downregulate cell surface expression of MHC class Ia molecules are deleted, Jones and Muzithras, (1992), showed that HLA-G expression was not rescued (Fisher *et al.*, 2000). Others reported that HLA-G is resistant to the effects of CMV protein US11, which binds to class I heavy chains and mediates their dislocation to the cytosol and subsequent proteasomal degradation (Schust *et al.*, 1998). Subsequent analyses using chimeric molecules of MHC class Ia and Ib showed that the degradation efficiency depended on sequences in the heavy-chain cytosolic tail that HLA-G lacks (Barel *et al.*, 2003). Since the mechanism of HLA-G downregulation does not involve CMV glycoproteins that alter class Ia expression, it is most likely novel.

CMV infection in vitro downregulates α1β1 integrin expression and impairs cytotrophoblast invasion

Congenital CMV infection is associated with abnormal placentation at a morphological level and intrauterine growth restriction (Benirschke *et al.*, 1974), likely related to impaired remodeling of uterine arterioles by invasive

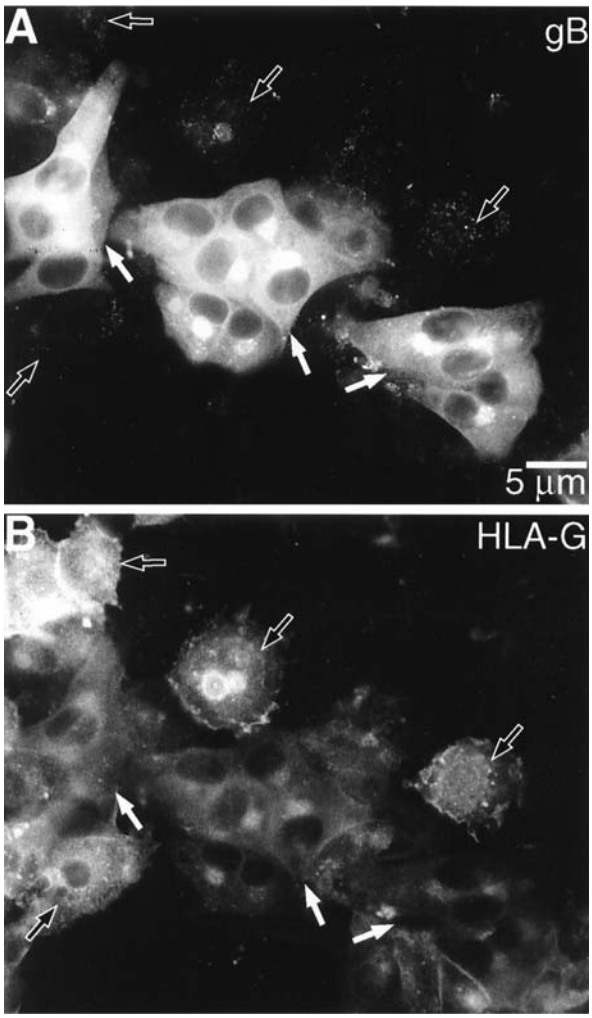

Fig. 45.5. CMV infection impairs cytotrophoblast expression of HLA-G in vitro. Purified cytotrophoblasts were isolated and infected with CMV. At 72 h, the cells were stained for (a) gB and (b) HLA-G expression. Cells that did not express gB (black arrows) expressed HLA-G, and staining for gB (white arrows) was associated with a marked reduction in HLA-G expression.

cytotrophoblasts. This prompted examination of the expression of the laminin/collagen receptor integrin α1β1 in the context of CMV infection. This extracellular matrix receptor is both a stage-specific antigen whose expression is preferentially associated with cytotrophoblasts inside the uterine wall (Damsky *et al.*, 1992) and an adhesion molecule that mediates invasion in vitro (Damsky *et al.*, 1994). Co-localization of CMV gB (Fig. 45.6(a) and (c)) and integrin α1 expression (Fig. 45.6(b) and (d)) showed that cells that did not stain for gB (Fig. 45.6(a)) expressed integrin α1 in a plasma membrane-associated pattern (Fig. 45.6(b)). Diffuse cytoplasmic staining for gB in infected cytotrophoblasts was also correlated with integrin α1 expression (see cell marked with an asterisk in Fig. 45.6(c) and (d)), but accumulation of gB in vesicles (Fig. 45.6(c)) at late times after infection was associated with the absence of staining for integrin α1 (Fig. 45.6(d)). In contrast, immunostaining for another integrin whose expression is upregulated as the cells invade, the fibronectin receptor α5 that functions to inhibit invasion, was not affected.

Flow cytometric analysis and RT-PCR were used to quantify proteins expressed on the surface of freshly isolated cytotrophoblasts from term placentas and changes in differentiating cells infected with VR1814, a pathogenic clinical strain (Tabata *et al.*, 2006). Significant downregulation of HLA-G at the protein level shown by immunohistochemistry was confirmed, and transcription was reduced in infected cells. Likewise, infected cytotrophoblasts significantly dysregulated integrin α1 and α5 proteins. Integrin α9 and VE-cadherin, which promote cell-cell adhesion, were also reduced by infection. Cytotrophoblasts isolated from placentas with CMV DNA and virion gB in syncytiotrophoblasts and in villus core macrophages were uninfected and showed similar expression of the differentiation molecules studied.

The impact of CMV infection on cytotrophoblast invasion was examined using an in vitro assay (see Fig. 45.2(b)) (Fisher *et al.*, 2000). This functional assay tests the ability of isolated cytotrophoblasts plated on the upper surfaces of Matrigel-coated filters to penetrate the surface, pass through pores in the underlying filter, and emerge on the lower surface of the membrane (Damsky *et al.*, 1994; Librach *et al.*, 1991). Invasion is quantified by determining the number of cytokeratin-positive cell processes that emerge through the filter pores. The invasion ability of cells infected with CMV was dramatically impaired, as compared with control uninfected cells, suggesting that functional defects could result from a constellation of virus-induced changes that impair cell–matrix and cell–cell adhesion. Interestingly, the effect on invasion was greater than could be accounted for by the number of CMV-infected cells, suggesting that the presence of infected cells in the invading aggregates influences the behavior of the population as a whole.

CMV infection downregulates MMP activity altering cell-cell and cell–matrix interactions

MMPs are a family of degradative enzymes that remodel the extracellular matrix during many processes, including cell migration, vascularization, and invasion (Chang and

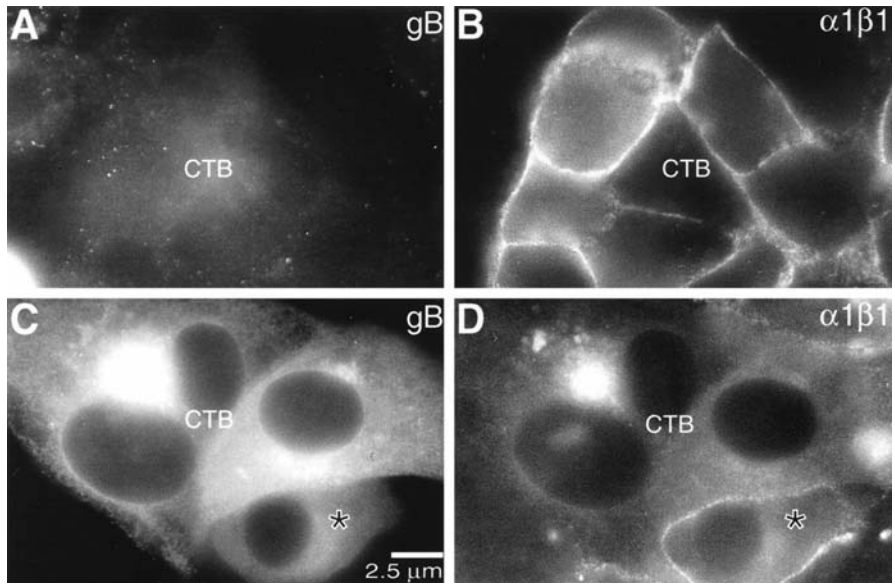

Fig. 45.6. CMV infection in vitro eventually downregulates cytotrophoblast expression of integrin α1. Purified cytotrophoblasts were infected with CMV in vitro. At 72 h, the cells were fixed and stained for expression of gB and integrin α1. Cytotrophoblasts that did not express gB (a) displayed prominent staining for integrin α1 in a plasma membrane-associated pattern (b). Likewise, cells that stained in a diffuse cytoplasmic pattern for gB (c) also reacted with the anti-integrin antibody (d, cell marked with an asterisk). However, when gB was localized in a vesicular pattern, integrin staining was not detected (d).

Werb, 2001). MMPs are highly regulated during translation and post-translationally by activation and secretion. Invasive cytotrophoblasts secrete relatively large amounts of MMP-9 in early gestation, when invasion peaks; later, when invasion is complete, MMP-9 levels fall (Librach *et al.*, 1991). Accordingly, cytotrophoblast invasion is also regulated by factors controlling MMP activation. The inactive proenzyme is activated by cleavage and removal of an inhibitory domain. Activated MMP-9 is absolutely required for invasion, whereas pro-MMP-9 is associated with non-invasive cells (Fisher *et al.*, 1989; Librach *et al.*, 1991).

Examination of cytotrophoblasts and endothelial cells infected with CMV in vitro showed altered MMP protein and activity (Yamamoto-Tabata *et al.*, 2004). Infection with VR1814, an endothelial cell-tropic CMV strain, but not AD169, a laboratory strain, reduced MMP-9 activity, thereby decreasing the cells' capacity to degrade the extracellular matrix. Likewise, MMP-2 activity in uterine microvascular endothelial cells was reduced. Since VR1814-infected endothelial cells transmit infection to cocultured differentiating cytotrophoblasts in vitro (Maidji *et al.*, 2002), infection could undermine contacts between endothelial cells and cytotrophoblasts (Fig. 45.2, site 1). The observation that uterine arterioles are infected by CMV in utero (Pereira *et al.*, 2003) suggests that virus could spread to cytotrophoblasts and in a retrograde direction to the placenta proper (i.e., floating chorionic villi) and to fetal blood vessels in the villus core.

CMV IL-10 dysregulates MMP activity

Several cytokines and growth factors regulate MMP expression and activity. For example, IL-1β is an autocrine stimulator of MMP-9 secretion and cytotrophoblast invasion of Matrigel in vitro (Librach *et al.*, 1994). In contrast, human IL-10 (hIL-10) downregulates these processes and impairs cytotrophoblast invasion (Roth *et al.*, 1996; Roth and Fisher, 1999). Recent reports indicated that CMV IL-10 (cmvIL-10) (Kotenko *et al.*, 2000) binds the hIL-10 receptor 1 (hIL-10R1) with affinity similar to that of natural ligand (Jones *et al.*, 2002) and has comparable immunosuppressive activity (Spencer *et al.*, 2002). Analysis of the cmvIL-10 genes from several strains showed very high sequence conservation, suggesting conserved functions (Kotenko *et al.*, 2000; Spencer *et al.*, 2002). Like other intracellular pathogens that infect macrophages, CMV exploits the IL-10 signaling pathway, expressing an IL10 homologue and upregulating the cell's production of the cytokine (Kotenko *et al.*, 2000; Redpath *et al.*, 2001). Although cmvIL-10 shares only 27% sequence identity with hIL-10, the proteins have essentially identical affinity for the receptor, IL-10R1, and similarly

reorganize the cell surface receptor complex (Jones *et al.*, 2002).

Both cytotrophoblasts and endothelial cells express IL-10R1, suggesting possible autocrine and paracrine regulation by its ligand (Cattaruzza *et al.*, 2003; Roth and Fisher, 1999). hIL-10 in cytotrophoblasts' culture medium can suppress allogeneic lymphocyte reactivity (Roth *et al.*, 1996), an important link between immune protection of the fetus and cytotrophoblast invasion of the uterus. Likewise, recombinant cmvIL-10 can inhibit proliferation of mitogen-stimulated peripheral blood mononuclear cells and production of proinflammatory cytokines at a level comparable to that of hIL-10 (Spencer *et al.*, 2002). Together these findings suggest that cmvIL-10 might, like the cellular molecule, impair invasion of differentiating cytotrophoblasts. MMP activity was examined in uterine microvascular endothelial cells and differentiating cytotrophoblasts in vitro treated with purified recombinant cmvIL-10 or hIL-10 (Yamamoto-Tabata *et al.*, 2004). Culture medium and cell lysates of treated endothelial cells contained less MMP activity than untreated contols, suggesting that the viral cytokine inhibits proteinase production in the absence of infection and that cmvIL-10-and hIL-10 have comparable effects. Likewise, levels of MMP-9 activity in differentiating cytotrophoblasts treated with these cytokines were significantly reduced in a dose-dependent fashion, confirming previous results (Roth and Fisher, 1999).

CMV IL-10 impairs endothelial cell migration and cytotrophoblast invasiveness in vitro

Having shown that cmvIL-10 downregulates MMP-2 and MMP-9 activity, the effect of reduced proteinase activity on fibroblast and endothelial cell function was examined in cell wound healing assays. Briefly, subconfluent cells were scratched ("wounded") and then incubated until control cells closed the wound. Infection with VR1814 or treatment with hIL-10 and cmvIL-10 had impaired endothelial cell wound closure but had no inhibitory effect on fibroblast migration. To assess the effect on cytotrophoblasts, the frequency with which the cells passed through narrow pores in a Matrigel-coated filter was quantified. Treatment with cmvIL-10 alone impaired invasion to a level comparable to that of hIL-10-treated cells, and significantly fewer cells traversed the filter pores after treatment with cmvIL-10 as compared with control untreated cells. Together these results indicated that, like hIL-10, cmvIL-10 impairs endothelial cell migration in wound closure assays and cytotrophoblast invasion as previously observed in CMV-infected cells in vitro (Fisher *et al.*, 2000). These studies suggest that CMV exploits an immune mechanism to dysregulate endothelial cell migration and cytotrophoblast invasion (Yamamoto-Tabata *et al.*, 2004).

Concluding remarks

We are just beginning to appreciate how the unusual anatomy of the maternal-fetal interface is advantageous for CMV spread to the placenta and how innate and adaptive immunity often precludes transplacental transmission. These studies open the door to testing a variety of hypotheses regarding CMV infection of placental tissues. Numerous questions and challenges remain. What is the functional significance of the static picture we obtained of immune defenses and viral proteins at the placental-decidual interface? Does coinfection with viruses and pathogenic bacteria in the decidua and adjacent placenta correlate with fetal transmission in early and late gestation? Additionally it will be interesting to decipher the network of cytokines and chemokines that regulates trafficking of immune cells in the infected decidua. Finally, identifying the molecules used for virion attachment and entry into cytotrophoblasts and syncytiotrophoblasts is crucial to the development of therapeutic strategies. One key to the puzzle of resolving infection *in utero* will be the capacity to gauge the threshold for maternal hyporesponsive. Onset of inflammation could trigger CMV reactivation and processes whereby NK cells, macrophages and dendritic cells control infection in the decidua. We theorize that detailed studies will resolve the serious dichotomy between the devastating consequences of congenital CMV infection and our lack of knowledge, at the molecular level, of the mechanisms involved.

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Part III

HHV-6A, 6B, and 7

46

HHV-6A, 6B, and 7: pathogenesis, host response, and clinical disease

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Human herpesvirus 6(HHV-6) is a human pathogen of emerging clinical significance. HHV-6 was first isolated from patients with lymphoproliferative disorders in 1986 (Salahuddin *et al.*, 1986). HHV-6 isolates are classified into two groups as variants A(HHV-6A) and variant B(HHV-6B) (Schirmer *et al.*, 1991. The two variants are closely related but show consistent differences in biological, immunological, epidemiological, and molecular properties. HHV-6B is the major causative agent of exanthem subitum (ES) (Yamanishi *et al.*, 1988), but no clear disease has yet been associated with HHV-6A.

Human herpesvirus 7 (HHV-7) was isolated in 1990 from a healthy individual whose cells were stimulated with antibody against CD3 and then incubated with interleukin-2 (Frenkel *et al.*, 1990). This virus is one of the causative agents of ES (Tanaka *et al.*, 1994). Therefore, HHV-6 and HHV-7 are also called Roseolovirus. HHV-6 and HHV-7 are ubiquitous, and more than 90% of adults have antibody to both viruses. These viruses have extensive homology and belong to the β-herpesvirus subfamily.

The genome of HHV-6A is 159 321 bp in size, has a base composition of 43% G + C, and contains 119 open reading frames. The overall structure is 143 kb bounded by 8 kb of direct repeats, DRL (left) and DRR (right), containing 0.35 kb of terminal and junctional arrays of human telomere-like simple repeats (Gompels *et al.*, 1995). A total of 115 potential open reading frames (ORFs) were identified within the 161 573-bp contiguous sequence of the entire HHV-6B genome (HST) (Isegawa *et al.*, 1999). The HHV-6B(Z29) genome is 162 114 bp long and is composed of a 144 528-bp unique segment (U) bracketed by 8793-bp direct repeats (DR). The genomic sequence allows prediction of a total of 119 unique open reading frames (ORFs), 9 of which are present only in HHV-6B. The overall nucleotide sequence identity between HHV-6A and HHV-6B is 90%. The most divergent regions are DR and the right end of the unique region, spanning ORFs U86 to U100. These regions have 85 and 72% nucleotide sequence identity, respectively (Dominguez *et al.*, 1999).

Virus entry and establishment of infection

Cell tropism in vitro

HHV-6A and HHV-6B replicate most efficiently in vitro in peripheral blood mononuclear cells (PBMCs) or cord blood lymphocytes (CBL), and several isolates have been adapted to grow efficiently in continuous T-cell lines. HHV-6 replicates in activated CD4 T lymphocytes in vivo. HHV-6A and HHV-6B differ in their capacities to replicate in specific transformed T-lymphocyte cell lines. Of the two most widely used strains of HHV-6A, strain GS is most commonly propagated in the T-cell line HSB-2 and strain U1102 is usually propagated in J JHAN cells. HHV-6B (Z29 or HST) is grown most often in primary lymphocytes and has been adapted for growth in the Molt-3 or MT-4 –T cells line. While T cells are most widely used for propagation of HHV-6A and HHV-6B, cell lines of neural, epithelial, and fibroblastic origin have different levels of permissiveness for HHV-6 growth in vitro. However, none of these cells are in general use for routine propagation of the virus. In patients with dual infection, only HHV-6A persisted in CSF, which suggests that HHV-6A has greater neurotropism (Hall *et al.*, 1998). Furthermore, CD8 T-lymphocytes, gamma/delta T-lymphocytes and natural killer (NK) cells support HHV-6 replication in association with surface expression of CD4 (Lusso *et al.*, 1991a,b, 1995; Hall *et al.*, 1998).

Grivel et al. showed that HHV-6A and HHV-6B replicate in human lymphoid tissue, but have significant differences in effects on cellular viability and immunological phenotype (Grivel *et al.*, 2003). There is productive infection of both

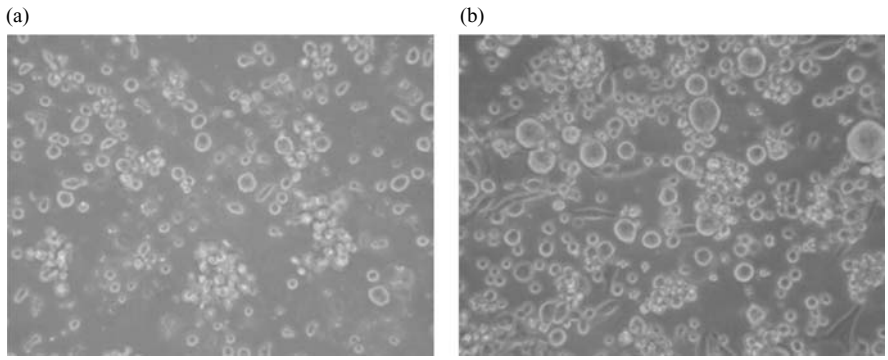

Fig. 46.1. Cytopathic effects of HHV-6A or HHH-7. (a) HSB-2 cells were infected with HHV-6A. (b) SupT1 cells were infected with HHV-7.

CD4+ and CD8+ T-cells, although HHV-6B is markedly less efficient than HHV-6A in targeting CD8+ T-cells. CD46 and CD3 are down-modulated in HHV-6 infected tissues. However CD3 down-modulation is restricted to infected cells, while the loss of CD46 expression is generalized. Thus, the down-modulation of CD46 in HHV-6 negative cells most likely represents an authentic bystander effect. Moreover, HHV-6 infection markedly enhanced the production of CC chemokine RANTES.

In contrast, HHV-7 has a narrow tropism for CD4+ T-cells, associated with infectivity for PHA-stimulated PBMCs, CBLs and in an immature T- cell line SupT1 (Cermelli *et al.*, 1997). Both HHV-6 and HHV-7 induce a cytopathic effect in infected cells which is characterized by ballooning degeneration (Fig. 46.1).

Cell tropism in vivo

The in vivo host tissue range of HHV-6 is broader than its in vitro host range might suggest and includes lymph nodes, lymphocytes, macrophages and monocytes, kidney tubule endothelial cells, salivary glands, and CNS tissues, where viral gene products have been localized to neurons and oligodendrocytes.

HHV-6 genomes and/or antigens are detetable in lymph nodes of patients with sinus histiocytosis with massive lymphadenopathy (SHML) (Levine *et al.*, 1992), tubular epithelial cells, endothelial cells and histiocytes in kidney (Kurata *et al.*, 1990), salivary glands (Fox *et al.*, 1990), and central nervous system (CNS) tissues, where viral gene products have been localized to neurons and oligodendrocytes (Luppi *et al.*, 1994). HHV-6 is also detected in lesions of Langerhans cell histiocytosis in the syndrome of Langerhans cell histiocytosis (Leahy *et al.*, 1993).

HHV-6 was isolated from CD4+ CD8− and CD3+ CD4+ mature T-lymphocytes but could not be isolated from CD4− CD8+, CD4− CD8−, and CD3− T-cells in the peripheral blood of exanthem subitum patients. HHV-6 predominantly infected CD4+ CD8+, CD4+ CD8-, and CD3+ CD4+ cells with mature phenotypes and rarely infected CD4- CD8+ cells from cord blood mononuclear cells, which suggested a predominant tropism of HHV-6 for mature CD4 T-lymphocytes (Takahashi *et al.*, 1989).

So far, two cell types have been recognized as sites of HHV-7 infection in vivo, including CD4+ T-lymphocytes and epithelial cells of salivary glands (Black *et al.*, 1993). HHV-7 is frequently isolated from saliva of healthy adults (Wyatt and Frenkel, 1992). A recent study showed that cells expressing the HHV-7 structural antigen were also detectable in lungs, skin, and mammary glands. Liver, kidney, and tonsils were also positive, although the number of HHV-7-positive cells was low. Large intestine, spleen, and brain were negative for HHV-7 infection (Kempf *et al.*, 1998).

Entry

HHV-6 is characterized by a broad tropism for human cell types but a narrow range of host species. Santoro *et al.* (1999) identified human CD46 as the cellular receptor for HHV-6. CD46 is a ubiquitous type 1 glycoprotein expressed on the surfaces of all nucleated human cells (Seya *et al.*, 1990). It was originally purified as a complement (C) regulatory protein; it binds C3b and C4b on host cells to allow factor I-mediated inactivation of these C fragments, and it plays an important role in protecting host cells from autologous C protein (Seya *et al.*, 1990). CD46 is also a cellular receptor for measles virus. Evidence for CD46 receptor activity included (i) A selective and progressive down-regulation of the surface membrane expression of CD46 in activated human CD4+ T-cells in the course of HHV-6A and HHV-6B infection, (ii) inhibition of HHV-6

infection and associated cell fusion by Mab against CD46 and by soluble CD46, and (iii) non-human cells being rendered susceptible to HHV-6-mediated membrane fusion and HHV-6 entry by expression of CD46. However, the expression of CD46 was not sufficient for HHV-6 fusion and infection in all human cell types, suggesting either that a specific co-receptor is needed or that some cells express an inhibitor of the CD46 receptor activity. Mori *et al.* (2002) found that HHV-6A, but not HHV-6B can mediate fusion-from-without (FFWO) in a variety of human cells, including Vero cells which are an old world monkey cell line. Chinese hamster ovary (CHO) cells are highly resistant to infection by HHV-6 and cell-cell fusion induced by HHV-6A. However HHV-6A, but not HHV-6B induced cell–cell fusion in CHO cells expressing human CD46 without virus replication. Thus, the induction of cell- cell- fusion in the target cells by HHV-6A requires human CD46. Thus, HHV-6A mediates syncytia formation in target cells expressing human CD46 without associated virus replication. Human CD46 is composed of four short consensus repeats (SCRs), a Ser/Thr(ST)-rich domain, 13-amino-acid sequence of unknown significance (UK), a transmembrane domain, and a cytoplasmic tail (CYT) (Seya *et al.*, 1990). The SCR2, -3 and - 4 of the CD46 ectodomain were essential for the HHV-6A induced cell-cell fusion. Another report indicates that the SCR domains 2 and 3 are required for HHV-6 receptor activity (Greenstone *et al.*, 2002).

The products encoded by the U100 gene of HHV-6A have been reported to form a complex containing polypeptides. The U100 gene complex is a major component of the HHV-6 virion and a target for virus-neutralizing antibodies. The gene has an intron-exon structure, resulting in a highly spliced mRNA transcript, and is unique to HHV-6 and HHV-7 (Pfeiffer *et al.*, 1995; Skrincosky *et al.*, 2001). U100 gene products of HHV-6A are mainly composed of 80- and 78-kDa glycoproteins and furthermore, the 80-kDa gene product is the third glycoprotein component of the gH-gL complex in HHV-6A infected cells (Mori *et al.*, 2003a,b). Based on these characteristics, U100 gene products were designated as glycoprotein Q(gQ). The gH-gL-gQ complex is identified as a viral ligand for human CD46 (Mori *et al.*, 2003a,b). The gH-gL complex alone or gQ alone in a transient expression system were unable to bind to CD46. The interaction with CD46 might require additional associations or modification in HHV-6 infected cells. Santoro et al. showed that gH of HHV-6 is a ligand for human CD46, however gH alone does not bind to CD46, and the interaction between gH and CD46 requires HHV-6 infection (Santoro *et al.*, 2003). Therefore, to date, whether one glycoprotein of the gH–gL–gQ complex binds to CD46 directly or whether the steric conformation of the complex itself is required for the interaction with CD46 is unknown.

The entry of herpes viruses into cells is a complex process that is still incompletely understood. In several cases, it appears to require not only a cellular receptor to interact with the virus attachment protein but also at least one additional molecule to interact with the virus and facilitate penetration. Studies on other herpesviruses have provided indirect evidence of a role for homologue of the gH-gL and gB molecules in membrane fusion. In HHV-6, specific monoclonal antibodies against glycoprotein H (gH) and glycoprotein B (gB) inhibited virus-induced cell fusion event and infection (Foa Tomasi *et al.*, 1991; Mori *et al.*, 2002). Considering the previous reports, it seems likely that the process involves several steps. First, HHV-6 gH-gL-gQ complex binds to CD46 and at the same time, gB binds to an unknown cellular molecule, thereby triggering fusogenic activity. Subsequently, viral envelope glycoproteins, probably gB and gH-gL-gQ may act to induce envelope–cell or cell–cell fusion. gB or gH or both are candidates for the actual fusogenic glycoprotens. HHV-7 infects CD4+ T-lymphocye in vitro. The glycoprotein CD4, a member of the immunoglobulin superfamily, is a critical component of the receptor for HHV-7 (Lusso *et al.*, 1994). A selective and progressive downregulation of the surface membrane expression of CD4 was observed in human CD4+ T-cells in the course of HHV-7 infection. Various murine monoclonal antibodies (MAbs) to CD4 and the recombinant soluble form of human CD4 caused a dose-dependent inhibition of HHV-7 infection in primary CD4+ T-lymphocytes. Moreover, radiolabeled HHV-7 specifically bound to cervical carcinoma cells (HeLa) expressing human CD4. However, HHV-7 can infect cells that do not express detectable CD4. It is likely that other host molecules act as receptors; the need for multiple sequential receptors to enable cell-to-cell migration of the virus in tissues is a well-documented phenomenon in other herpesviruses.

The human immunodeficiency virus type 1 (HIV-1) co-receptors, CXC-chemokine receptor (CXCR)4 and CC-chemokine receptor(CCR)5, have been studied to determine whether they serve similar functions for HHV-6A, HHV-6B and HHV-7. Cells from individuals lacking CCR5 were able to support growth of all three viruses, and these individuals were seropositive for the viruses, indicating that this molecule is not essential for viral replication. HHV-7 infection also causes a progressive loss of the surface CXCR4 in CD4(+) T-cells, accompanied by a reduced intracellular Ca2+ flux and chemotaxis in response to stromal cell-derived factor-1 (SDF-1), the specific CXCR4 ligand. Moreover, CXCR4 is downregulated from the surface of HHV-7-infected T-cells independently of CD4. Because

intracellular CXCR4 antigen and mRNA levels are unaffected in productively HHV-7-infected cells, the down-regulation of CXCR4 apparently does not involve a transcriptional block (Secchiero *et al.*, 1998). However, another report demonstrates that CXCR4 is not involved in HHV-7 infection. The natural ligand of CXCR4, SDF-1alpha, was not able to inhibit HHV-7 infection in SupT1 cells or in CD8(+) T-cell-depleted peripheral blood mononuclear cells. Also, a specific CXCR4 antagonist with potent antiviral activity against T-tropic HIV strains (50% inhibitory concentration IC(50), 1 to 10 ng/ml), completely failed to inhibit HHV-7 infection (IC(50), >250 μ/ml) (Zhang *et al.*, 2000). Unlike HIV-1, HHV-6 and HHV-7 infections do not require expression of CXCR4 or CCR5, whereas marked down-regulation of CXCR4 is induced by these viruses (Yasukawa *et al.*, 1999).

Two HHV-7 glycoproteins have been identified as being able to bind the cell surface proteoglycans heparan and heparan sulfate (Secchiero *et al.*, 1997a,b; Skrincosky *et al.*, 2001). They are the virion glycoprotein, gB and spliced glycoprotein encoded by U100. Thus, soluble heparin was found to block HHV-7 infection and syncytium formation in the SupT1 cell line. The CD4 antigen is a critical component of the receptor for the T-lymphotropic HHV-7 suggesting that heparin-like molecules also play an important role in the HHV-7-entry process. As described above, gB is one of the HHV-7 envelope proteins involved in the adsorption of virus-to-cell surface proteoglycans (Secchiero *et al.*, 1997a,b). Analysis of the biochemical properties of recombinant gp65, (U100 gene products), also revealed a specific interaction with heparin and heparan sulfate proteoglycans and not with closely related molecules such as N-acetylheparin and de-N-sulfated heparin, suggesting that HHV-7 gp65 may contribute to viral attachment to cell surface proteoglycans (Skrincosky *et al.*, 2001). The products of U100 are targets for complement-independent neutralization.

Envelope glycoproteins for entry process

The genes U39 and U48 of HHV-6 and HHV-7 encode the conserved surface glycoproteins gB and gH, which contribute to virus-cell fusion.

HHV-6 gH forms complexes with glycoprotein L (gL, encoded by U82), resulting in the formation of a gp100 complex (Liu *et al.*, 1993). Recently, gQ (encoded by U100) was shown to be a third component of gH-gL complex in HHV-6 (Mori *et al.*, 2003a,b). This gQ is unique to the genus of HHV-6 and HHV-7. The gQ gene is subject to differential splicing, and a number of enveloped glycoprotein –encoding genes, gQ genes of HHV-6A and –6B demonstrate only 72.1% sequence identity. This glycoprotein may have a role in the differential consequences of HHV-6A and B infections. Along with gB and gH, gQ contains epitopes recognized by variant specific neutralizing antibodies.

An unusual feature of HHV-6 in comparison to other herpesviruses is the lack of viral glycoproteins in the plasma membrane (Cirone *et al.*, 1994). HSB-2 T-lymphoid cells and human cord blood mononuclear cells infected with HHV-6 reveal the presence, in the cell cytoplasm, of annulate lamellae (AL), which are absent in uninfected cells (Cardinali *et al.*, 1998). Viral glycoproteins are stored in newly formed annulate lamellae, which function as a viral glycoprotein storage compartment and as a putative site of O-glycosylation. It is proposed that, during viral morphogenesis, nucleocapsids released from the nucleus have a primary envelope that lacks glycoproteins but, in the cytoplasm, this is removed and replaced by a secondary envelope containing glycoproteins acquired from the annulate lamella. Further modification of glycoproteins by glycosylation during transit through the Golgi apparatus occurs before mature virions are released.

Spread in host, mechanisms of tissue damage

Growth properties

HHV-6 and HHV-7 replication cycles are approximately 3 days in activated CBLs grown in the presence of IL-2or PHA. Even in most permissive systems, the infectious yields are relatively low, commonly ranging from 10^3 to 10^5 infectious units per ml. Centrifugal infection increases the infectious titer.

Effects of virus infection on host cells

HHV-6 infection has profound effects on host cells. These lead to the development of the classic cytopathic effect of ballooning and multinucleated giant cells.

In the case of HHV-7, multinucleated giant cells occur, not by fusion of cells into syncytia, but by polyploidization (Secchiero *et al.*, 1998). The giant cells, which represent the hallmark of in vitro HHV-7 infection, arise from single CD4(+) T-cells undergoing a process of polyploidization that is linked to disregulation of cyclin-dependent kinase cdc2 and cyclin B. This leads to an accumulation of cells in the G_2 to M phase of the cell cycle, with nuclei continuing to reproduce in the absence of cell division (Secchiero *et al.*, 1998).

Several cytokines can be induced by HHV-6 and HHV-7 infection. Interferon-alpha, interleukin 1 beta, and tumor necrosis factor are induced by HHV-6 (Kikuta *et al.*, 1990; Flamand *et al.*, 1991). But, exposure of human macrophages to HHV-6 profoundly impairs their ability to produce IL-12 upon stimulation with IFN-gamma and LPS, providing a novel potential mechanism of HHV-6-mediated immunosuppression (Smith *et al.*, 2003). HHV-6 can infect NK cells and T lymphocytes. HHV-6 and HHV-7 induces IL-15 in human PBMC and increases their NK activity (Flamand *et al.*, 1996; Atedzoe *et al.*, 1997; Gosselin *et al.*, 1999). The induction of NK cell activity by HHV-6 is abrogated by monoclonal antibodies to IL-15 but not by mAbs to other cytokines (IFN-alpha, IFN-gamma, TNF-alpha, TNF-beta, IL-2, IL-12). IL-15 protein synthesis is increased in response to HHV-6, and addition of IL-15 to PBMC cultures is found to severely curtail HHV-6 expression. Taken together, the host responds to HHV-6 and HHV-7 infection by up-regulating IL-15 production, which then results in an enhancement of NK cell activity; this, in turn, may play a major role in the control of the viral infection (Flamand *et al.*, 1996; Atedzoe *et al.*, 1997; Gosselin *et al.*, 1999).

HHV-6 affects HIV-1 infection in a coreceptor- dependent manner, suppressing CCR5-tropic but not CXCR4-tropic HIV-1 replication. HHV-6 increases the production of the CCR5 ligand RANTES CC-chemokine, the most potent HIV-inhibitory CC chemokine, and that exogenous RANTES mimics the effects of HHV-6 on HIV-1, providing a mechanism for the selective blockade of CCR5-tropic HIV-1 (Grivel *et al.*, 2001). HHV6 infection induces de novo synthesis of the RANTES in endothelial cells as well (Caruso *et al.*, 2003).

HHV-6A infection induces cell-surface expression of CD4, which then allows infection by HIV-1 of cells such as gamma/delta T cells that were previously refractile to infection (Lusso *et al.*, 1991a,b, 1995; Caruso *et al.*, 2003). HHV-6A, but not HHV-6B or HHV-7, down-regulates cell surface expression of CD3, and HHV-7 predominantly down-regulates CD4 (Furukawa *et al.*, 1994).

HHV-6A, HHV-6B and HHV-7 were evaluated for their effects on in vitro colony formation of hemopoietic progenitor cells derived from CBLs. Formation of both granulocyte/macrophage and erythroid colonies was suppressed after infection with HHV-6B. Although HHV-6A suppressed the formation of erythroid colonies as efficiently as HHV-6B, HHV-6A did not exhibit significant suppressive effect on the formation of granulocyte/macrophage colonies. HHV-7 had no effect on either lineage (Isomura *et al.*, 1997). Furthermore, the suppressive effects of HHV-6 on thrombopoiesis in vitro was evaluated. Using CBLs as the source of hematopoietic progenitors, two types of colonies, megakaryocyte colony-forming units and non-megakaryocyte colony-forming units colonies, were established. HHV-6A and HHV-6B inhibited thrombopoietin-inducible both megakaryocyte and non-megakaryocyte colony formation. In contrast, HHV-7 had no effect on thrombopoietin-inducible- colony formation (Isomura *et al.*, 2000). More differentiated CD34+ cells, which were a major source of hematopoietic progenitor cells, were more susceptible to the effects of HHV-6, indicating that the targets for hematopoietic suppression by HHV-6 are the differentiated cells (Isomura *et al.*, 2003). In contrast, in bone marrow-derived cells, both HHV-6A and HHV-6B suppressed erythroid, granulocyte-macrophage, and multipotential precursors of the granulocyte, erythrocyte, monocyte, and megakaryocyte lineages (Carrigan and Knox, 1995). The mechanisms of cell death in the human CD4+ T-cell line J JHAN mediated by HHV-6 were investigated (Inoue *et al.*, 1997) by transmission electron microscopy infected cells showed characteristics of apoptosis, such as chromatin condensation and fragmentation of nuclei, but few virus particles were detected in apoptotic cells. Two-color flow cytometric analysis revealed that DNA fragmentation was present predominantly in uninfected cells but not in cells that were productively infected with HHV-6 (Inoue *et al.*, 1997). Acute in vitro HHV-7 infection induced (i) the formation of giant multinucleated syncytia, which eventually underwent necrotic lysis, and (ii) single-cell apoptosis. Using electron microscopy analysis, all syncytia contained large amounts of virions and most cells within syncytia them exhibited clear evidence of necrosis, whereas apoptosis was predominantly observed in single cells. Although empty viral capsids could be identified in the cytoplasm of approximately 25% of single cells exhibiting an apoptotic morphology, few mature virions were observed in these cells. Thus, it appears that apoptosis occurred predominantly in uninfected bystander cells but not in productively HHV-7-infected cells (Secchiero *et al.*, 1997a,b). Apoptosis induced by HHV-6 in cord blood mononuclear cells (CBMCs) was also investigated. CBMCs prestimulated with phytohemagglutinin (PHA) were infected with HHV-6 and cultured with interleukin 2 (IL-2) for 5 days. The percentage of the hypodiploid fraction by cell cycle analysis and the percentage of cells showing apoptosis determined by terminal deoxytransferase (TdT)- mediated dUTP nick end-labeling (TUNEL) assay were significantly higher in HHV-6-infected CBMC compared with uninfected CBMC. 7A6 antigen, induced on the mitochondria membrane in apoptotic cells, was mainly expressed in CD4+ cells; 7A6 antigen was also detected in HHV-6-infected cells as determined by expression of gH.

Thus, HHV-6 induces apoptosis in HHV-6-infected CBMCs different from T-cells lines (Ichimi *et al.*, 1999). In order to confirm that apoptosis of CD4+ T lymphocytes also occurs in HHV-6 infection in vivo, apoptosis of lymphocytes isolated from nine patients with exanthem subitum and from an adult patient with severe HHV-6 infection was examined (Yasukawa *et al.*, 1998). PBMCs were cultured for 3 days and apoptosis of lymphocytes was examined by flow cytometry of propidium iodide-stained DNA. The percentages of hypodiploid DNA, indicating apoptosis, in lymphocytes from 10 patients with HHV-6 infection were significantly higher than those from five infant patients with non-infectious diseases and five healthy adults ($P < 0–0002$). DNA fragmentation was also detected in lymphocytes from patients with HHV-6 infection. Apoptosis appears to occur predominantly in CD4+ T-lymphocytes and HHV-6 is isolated from the CD4+ T lymphocyte fraction (Yasukawa *et al.*, 1998).

Accordingly, in CBLs, infected cells are apoptotic, while in transformed cells, infected cells die by necrotic lysis and apoptosis is triggered in non-productively infected cells. The latter observation suggests that the virus may be able to inhibit apoptosis in at least some cells and that its replication might be enhanced by suppression of apoptosis.

To dissect the underlying molecular events, the role of death-inducing ligands belonging to the tumor necrosis factor (TNF) cytokine superfamily was investigated (Secchiero *et al.*, 2001a,b). HHV-7 selectively up-regulated the expression of TNF-related apoptosis-inducing ligand (TRAIL), but not that of CD95 ligand or TNF-alpha in SupT1 or primary activated CD4(+) T-cells. Moreover, in a cell-to-cell-contact assay, HHV-7-infected CD4(+) T-lymphocytes were cytotoxic for bystander uninfected CD4(+) T-cells through the TRAIL pathway. By contrast, HHV-7 infection caused a marked decrease of surface TRAIL-R1, but not of TRAIL-R2, CD95, TNF-R1, or TNF-R2. Of note, the down-regulation of TRAIL-R1 selectively occurred in cells coexpressing HHV-7 antigens that became resistant to TRAIL-mediated cytotoxicity. These data suggest that the TRAIL-mediated induction of T-cell death may represent an important immune evasion mechanism of HHV-7, helping the virus to persist in the host organism throughout its lifetime (Secchiero *et al.*, 2001a,b).

Disease consequences

Clinical features in hosts

Primary infection

Both HHV-6 and HHV-7 are ubiquitous viruses, and infection occurs during infancy. HHV-6B is a causative agent of ES (Yamanishi *et al.*, 1988). In most cases, ES is benign; it is associated with other symptoms including diarrhea, cough, lymph node swelling as bulging fontanel. ES is a common disease of infants all over the world. Typically, the infant gets sudden fever, which lasts for a few days, and a rash appears on the trunk and face and spreads to the lower extremities as the fever subsides. In adults, primary infections can cause mononucleosis like disease and hemophagocytic syndrome (Akashi *et al.*, 1993). HHV-7 can also cause ES and was isolated from PBMCs of a infant with typical ES (Tanaka *et al.*, 1994). The median age of children with primary HHV-7 infection was 26 months, which is significantly older than that of children with primary HHV-6 infection (median, 9 months).

Immune response during primary infection

The early immune response was studied by assessing interferon (IFN) and natural killer cell activity in 13 patients with ES associated with HHV-6 infection during the acute and convalescent phases (Takahashi *et al.*, 1992). Only IFN-alpha was significantly increased in the plasma of patients during the acute febrile phase compared with the convalescent period. The inhibitory effect of IFN-alpha and IFN-beta on HHV-6 replication was demonstrated in vitro with cord blood mononuclear cells. Natural killer cell activity was also significantly augmented in the acute phase, especially in the exanthem period, compared to in the convalescent phase. These results suggest that the enhanced IFN-alpha response and natural killer cell activity in the acute early phase of the disease may play pivotal roles in the recovery from ES.

Other symptoms associated with primary HHV-6 and 7 infection

The primary infection by HHV-6 and HHV-7 can cause a highly febrile illness in childhood, complicated by seizures (Torigoe *et al.*, 1996). Cases of possible HHV-6-associated encephalitis in young children have been reported (Asano *et al.*, 1992). Self-limited involvement of the central nervous system (CNS) is a relatively common complication of primary infection with HHV-6 in normal children. Liver dysfunction (Asano *et al.*, 1990; Tajiri *et al.*, 1990), idiopathic thrombocytopenic purpura (Yoshikawa *et al.*, 1993) are also associated with HHV-6 infection.

Reactivation of HHV-6 and its clinical symptoms

Since HHV-6 and HHV-7 establish latency following primary infection, they are important pathogens in immunocompromised hosts. Reactivation of HHV-6 and HHV-7 ocurres in patients after bone marrow transplantation,

solid organ transplantation such as liver, renal and heart transplantation, and AIDS.

Bone marrow transplantation (BMT)

Asymptomatic HHV-6 reactivations appear to be common following allogeneic BMT (Cone *et al.*, 1999), but HHV-6 reactivation associated with symptoms such as bone marrow suppression, encephalitis (Drobyski *et al.*, 1994; Tsujimura *et al.*, 1998; Rodrigues, 1999), pneumonitis (Cone *et al.*, 1993) and acute graft-versus-host disease (GVHD) in BMT recipients has also been recognized. Idiopathic marrow suppression occurred frequently in patients with concurrent HHV-6 viremia (Drobyski *et al.*, 1993). Infection with HHV-6 has been correlated with the development of skin rashes. HHV-6 DNA was detected in skin and/or rectal biopsies more frequently in allogeneic recipients with severe GVHD (92%) than in those with either moderate (55%) or mild GVHD (22%), suggesting that the presence of HHV-6 DNA in the skin or rectum may be a factor in determining GVHD severity (Appleton *et al.*, 1995).

Solid organ transplantation

HHV-6 infection after liver transplantation is associated with an immunosuppressive state (Singh *et al.*, 1995, 2002a,b). Acute febrile illness characterized by life-threatening thrombocytopenia, progressive encephalopathy and skin rash occurred with invasive HHV-6 infection in a liver transplant recipient (Singh *et al.*, 1995). Prolonged suppression of the HHV-6 memory response, but not overall T-helper cell function was documented and may play a role in the pathogenesis of HHV-6 infection in liver transplant recipients (Singh *et al.*, 2002a,b). The memory response to CMV after liver transplantation was significantly more robust than to HHV-6 (Singh *et al.*, 2002a,b). Griffiths et al. conducted a prospective study of the possible relationship of HHV-6 and HHV-7 infection with clinical symptoms after liver transplantation (Griffiths *et al.*, 1999). Although the virus load for HCMV was significantly greater than that for HHV-6 or HHV-7, HHV-6 and HHV-7 may be the cause of some episodes of hepatitis and pyrexia (Griffiths *et al.*, 1999). HHV-6 and CMV are significantly and independently associated with biopsy-proven graft rejection after liver transplantation (Griffiths *et al.*, 2000).

AIDS

Reactivation of HHV-6 has been reported to be possibly associated with interstitial pneumonia, encephalitis, and retinal disorder in AIDS patients, but specific clinical syndromes associated with reactivation are rare.

The other possible associated diseases

Multiple sclerosis (MS)

Several studies have suggested an association between HHV-6 and MS (Challoner *et al.*, 1995; Soldan *et al.*, 1997). However, negative results were also seen in other reports (Coates and Bell, 1998). There was no significant difference between MS patients and non-MS-patients by staining brains immunocytochemically (Coates and Bell, 1998). Therefore, whether HHV-6 contributes to MS pathogenesis in unclear.

Drug hypersensitivity

Drug-induced hypersensitivity syndrome is characterized by a severe, potentially fatal, multi organ hypersensitivity reaction that usually appears after prolonged exposure to certain drugs. Its delayed onset and clinical resemblance to infectious mononucleosis suggest that underlying viral infections may trigger and activate the disease in susceptible individuals receiving these drugs. Reactivation of HHV-6, possibly in concert with HHV-7 may contribute to the development of a severe drug-induced hypersensitivity syndrome (Suzuki *et al.*, 1998; Tohyama *et al.*, 1998).

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HHV-6A, 6B, and 7: molecular basis of latency and reactivation

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Introduction

The human β-herpesvirus subfamily consists of human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7). HHV-6 and HHV-7 belong to the Roseolovirus genus of the β-herpesviruses, and the HHV-6 species are divided into two variants: HHV-6A and HHV-6B. These viruses establish a lifelong infection of their host, reactivate frequently, and reactivated viruses are shed into the saliva (Jordan, 1983; Krueger *et al.*, 1990). Some evidence suggests that the molecular mechanisms of viral latency and reactivation are shared among these viruses. HHV-6B is reactivated from latency after coinfection with HHV-7 (Katsafanas *et al.*, 1996), and HCMV disease is frequently associated with concurrent HHV-6 and HHV-7 reactivation in transplant patients (Lautenschlager *et al.*, 2000; Mendez *et al.*, 2001)

The sites of these viruses during latency are not completely defined. For HHV-6B, viral DNA is detected predominantly in the peripheral blood monocytes/macrophages of seropositive healthy adults (Kondo *et al.*, 1991). Furthermore, primary cultured macrophages support latent HHV-6B infection, and viral reactivation is induced in them by treatment with 12-0-tetradecanoylphorbol-13-acetate (TPA) (Kondo *et al.*, 1991). HHV-6B also establishes latency in myeloid cell lines (Yasukawa *et al.*, 1999), and that HHV-6B is detectable in CD34 (+) peripheral blood progenitor cells (Luppi *et al.*, 1999). Therefore, HHV-6B appears to establish latency in hematopoietic progenitor cells.

HHV-6A is detectable in the peripheral blood of seropositive adults (Drobyski *et al.*, 1993); however, a cell population that might harbor latent HHV-6A has not been identified. Unlike HHV-6B, HHV-6A does not establish latency in cultured macrophages (K. Kondo *et al.*, unpublished data).

Some evidence suggests that latent HHV-6 infection in the brain may be involved in the pathology of certain neurological diseases, such as recurrent febrile figures (Hall *et al.*, 1994; Kimberlin and Whitley, 1998; Kondo *et al.*, 1993), multiple sclerosis (MS) (Challoner *et al.*, 1995; Sola *et al.*, 1993) and encephalitis (Caserta *et al.*, 1994). However, the site of HHV-6 latency in the brain has not been identified.

HHV-7 can be reactivated from latently infected peripheral blood mononuclear cells by T-cell activation, and it was first isolated from the purified CD4 (+) T-cells of a healthy individual; however, the range of cell types in which HHV-7 can establish true latency is not clear (Frenkel *et al.*, 1990; Katsafanas *et al.*, 1996). A variety of tissues contain infected cells at a late stage of HHV-7 infection, suggesting that HHV-7 might cause a persistent infection rather than a true latent infection (Kempf *et al.*, 1997a). Since HHV-6B can be recovered after the latently infected cells are superinfected with HHV-7, these viruses may use similar mechanisms to maintain their latency (Katsafanas *et al.*, 1996).

The investigation of latency-associated transcripts is important for understanding the molecular basis of herpesvirus persistence; however, the latency-associated transcripts of HHV-6A and HHV-7 have not yet been identified. In this chapter, we discuss the molecular mechanisms of HHV-6B latency and reactivation that are suggested based on its latency-associated transcripts (Kondo *et al.*, 2002, 2003b).

Latency-associated transcripts of HHV-6

Two types of HHV-6B latency-associated transcripts (H6LTs) have been identified in the gene locus of the immediate early (IE) 1/2 genes (Kondo *et al.*, 2002). They are detected only in latently infected cells in vitro and in vivo. Although they are encoded in the same direction as the immediate early (IE) 1/2 genes and share their

protein-coding region with IE1/2, their transcription start sites and exon(s) are different from those of the productive-phase transcripts (Mirandola *et al.*, 1998; Schiewe *et al.*, 1994) (Fig. 47.1). Type I H6LTs originate at the latent start site (LSS) 1, which is located 9.7 kilobases upstream of the IE1/2 start site, and Type II H6LTs originate at LSS2, which is located between exons 2 and 3 of IE1/2 (Fig. 47.1). In addition, novel short ORFs with latency-associated exons are encoded at the 5 proximal region of the H6LTs (ORF99, ORF142, and ORF145 in Fig. 47.1) (Kondo *et al.*, 2002).

The structures of the H6LTs are similar to those of HCMV latency-specific transcripts (Kondo and Mocarski, 1995; Kondo *et al.*, 1996); the latter encode IE1/IE2 ORFs, and short ORFs appear in the latency-specific exon. Furthermore, in the case of the HCMV latent transcripts, the translation of the IE1/IE2 protein is probably prevented by the existence of latency-specific ORFs upstream of the IE1/IE2 ORFs. Similarly, the HHV-6 IE1/IE2 protein is not detectable in latently infected macrophages. These findings suggest that viral replication of HCMV and HHV-6 may be suppressed at the point of translation of the major immediate early proteins during latency. The function of these upstream ORFs is discussed below.

Consistent with these findings, HHV-6 and HCMV exhibit similarities in their latent infections: (i) Both viruses can establish latency in cells of the monocyte/macrophage lineage (Kempf *et al.*, 1997b; Taylor-Wiedeman *et al.*, 1991); (ii) both viruses tend to persist in the latent state but are reactivated frequently, and the reactivated viruses are shed into the saliva (Jordan, 1983; Krueger *et al.*, 1990); (iii) methylation of the immediate-early gene locus is observed similarly in HHV-6 and HCMV, suggesting that the latent viral genome of these two viruses is locally methylated (Gompels *et al.*, 1995; Honess *et al.*, 1989); and (iv) viral reactivation of HHV-6 is associated with HCMV reactivation (DesJardin *et al.*, 1998; Humar *et al.*, 2000).

The HHV-6 late gene U94, which is a homologue of the adeno-associated virus type 2 (AAV-2) rep gene (Rapp *et al.*, 2000; Thomson *et al.*, 1991), has also been reported to be expressed during latency (Rotola *et al.*, 1998). Because other human β-herpesviruses, such as HCMV and human herpesvirus 7, do not encode homologues of U94 in their genomes (Chee *et al.*, 1990; Megaw *et al.*, 1998; Nicholas, 1996) the U94 gene may play some role that is specific to HHV-6. The Rep protein of AAV-2 is a site-specific endonuclease and helicase that is involved in site-specific integration of the viral genome into the host genome. Chromosomally integrated HHV-6 DNA has been observed in lymphomas (Luppi *et al.*, 1993), and the integrated viral DNA is reportedly transmitted stably in the germ line (Daibata *et al.*, 1998). These findings suggest that the U94 gene may relate to HHV-6 integration. However, no evidence has been reported so far to support this possibility.

Gene regulation of latency-associated transcripts

In the gene regulation of the latency-associated transcripts, a similarity between HHV-6 and HCMV has been observed using a recombinant HHV-6 (Kondo *et al.*, 2003b). The recombinant virus has an enhanced green fluorescent protein-puromycin gene cassette containing the CMV major immediate-early promoter (HCMV-MIEP) (Fig. 47.2). Neither viral replication in T-cells nor latency/reactivation in macrophages is impaired in this recombinant virus. During HHV-6 latency, however, no expression of EGFP driven by the HCMV-MIEP is detected (Fig. 47.3(A)).

Gene expression from the HCMV-MIEP was investigated using the 5'-rapid amplification of cDNA ends (RACE) method (Fig. 47.3(B)), which showed the EGFP mRNA is transcribed from the latent infection transcription start sites (LSSs) 1 and 2 of HCMV (Fig. 47.3(C)), which are used to express the latency-associated transcripts of HCMV (Kondo *et al.*, 1996). The finding that the HCMV MIEP showed a latency-associated activity in the context of HHV-6 latency suggested that the transcriptional control of HHV-6 latency may share some common mechanism with that of HCMV latency (Kondo *et al.*, 2003a).

First molecular event of HHV-6 reactivation

To identify the first molecular event of HHV-6 reactivation, the latent infection system of HHV-6 was used. In this system, macrophages are infected with HHV-6B and cultured for 4 weeks. At 4 weeks postinfection, macrophages show no signs of viral replication, such as viral protein expression or infectious virus production. Viral reactivation is induced by treatment with TPA (20 ng/ml) for 7 days (Kondo *et al.*, 1991).

At the early stage of the induction (3 and 5 days after the treatment with TPA), the proportion of cells that express the type I H6LTs significantly increases; however, transcription of productive-phase IE1/IE2 is not detected at days 0, 3, and 5 (Fig. 47.4). At this phase, IE1 protein is detectable in the cells without the production of infectious virus. Because productive-phase IE1 mRNAs are not detectable, the H6LTs, which contain the IE1 ORF (Fig. 47.1), are thought to be translated into IE1 protein at the first stage of viral reactivation. A similar molecular event has been

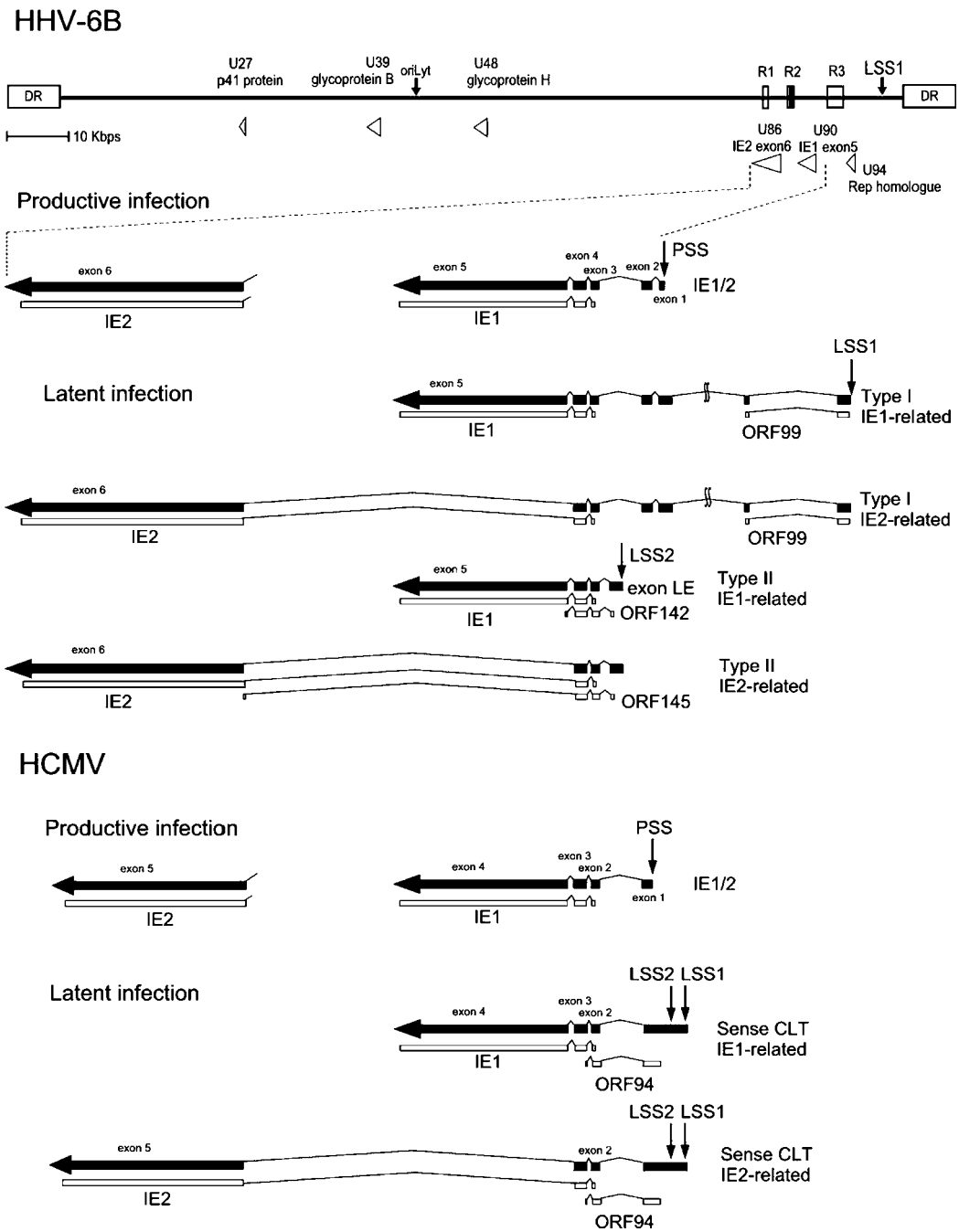

Fig. 47.1. Structures of the HHV-6 and HCMV latency-associated transcripts. Schematic drawings of the H6LT structures are shown. Productive-phase transcripts are also shown. The drawings of the mRNAs are in the same orientation relative to the viral genome. Thin lines represent introns; thick arrows represent exons. All exons and introns are drawn to scale. Latency-associated exons starting from latent start site (LSS) 1 and LSS2 are depicted. The position of the productive start site (PSS) is also shown. In HHV-6, exon 1 of the type I latent transcript is 138-bp longer than that of IE1/2. Two additional exons of the type I latent transcripts are located approximately 7.8 kb and 9.7 kb upstream from the PSS. ORFs of IE1, IE2, and putative latency-associated proteins ORF99, ORF142, and ORF145 are depicted. In HCMV, exon 1 of the cytomegalovirus latency-associated transcript (CLT) is longer than that of IE1/2. Latency-associated exons starting from LSS1 and LSS2 are depicted. ORFs of IE1, IE2, and putative latency-associated proteins ORF94 are depicted.

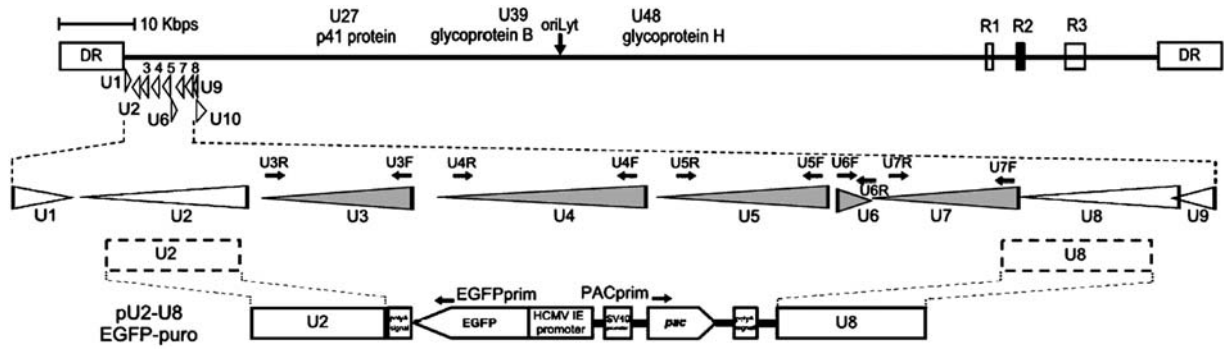

Fig. 47.2. Structure of recombinant HHV-6. At the top is a map of the HHV-6B genome, with the region U1-U9 expanded below. In the middle, shaded arrows show the U3-U7 ORFs that were replaced by the EGFP-puro cassette. The bottom diagram represents the EGFP-puro cassette pU2-U8 EGFP-puro. The open box represents the EGFP gene and human cytomegalovirus major immediate-early promoter. The puromycin-N-acetyl-transferase gene (*pac*) and SV40 early promoter are depicted.

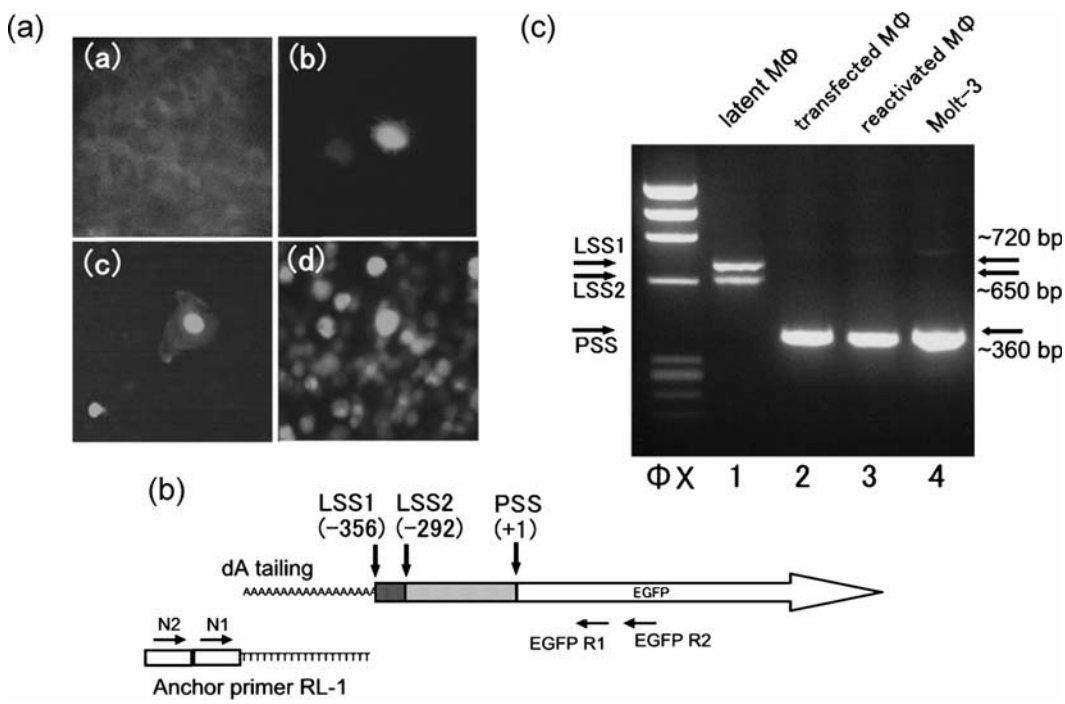

Fig. 47.3. (a) EGFP expression in various types of cells. Live cultured cells were observed under fluorescence. (a) Macrophages that were latently infected with EGFP-HHV-6; (b) latently infected macrophages that were(transfected with plasmid pU2-U8 EGFP-puro shown in Fig. 47.2(c) reactivation-induced macrophages; (d) productively infected Molt-3 cells.
(b) HCMV IE1/IE2 promoter and PCR primers. The EGFP gene and transcription start sites are drawn to scale. The productive infection transcription start site of IE1/IE2 mRNA (PSS indicated as +1) and two latent infection transcription start sites (LSS1 and LSS2: ref. 21) are shown. The locations of the PCR primers are depicted, and a schematic drawing shows the usage of the anchor primer RL-1.
(c) 5'-RACE amplification of the EGFP transcripts. RNA from latently infected macrophages (Mφ) (lane 1), latently infected macrophages that were transfected with plasmid pU2-U8 EGFP-puro shown in Fig. 47.2 (lane 2), reactivation-induced macrophages (lane 3), and productively infected Molt-3 cells (lane 4), was analyzed by the 5'-RACE method. The 5'-end of the transcript initiating at each of PSS (~360 bp), LSS1 (~720 bp), and/or LSS2 (~650 bp) was detected. Hae III-digested φX174 DNA fragments were used as size markers (φX).

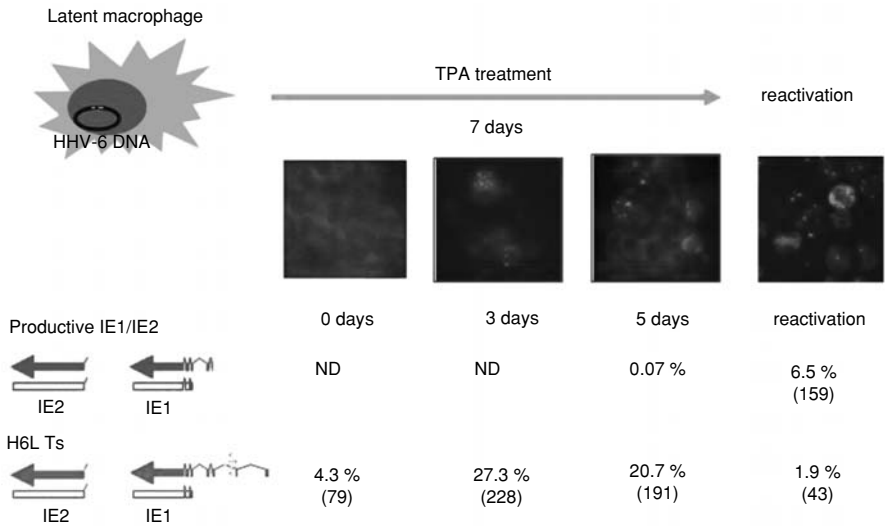

Fig. 47.4. mRNA and protein expression in latently infected macrophages. Latently infected macrophages were treated with TPA for 3, 5, and 7 days (reactivation). Cells were then stained with a mono-specific antibody against IE1. Percentage of H6LT-expressing cells during viral latency and reactivation. The percentages of H6LT-positive cells during viral reactivation were estimated. The copy number of each type of transcript in one H6LT-positive cell is shown in parentheses. The percentage of cells that showed the productive IE1/IE2 is also shown.

observed in transplant patients: approximately two-thirds of reactivation-positive patients express the type I H6LTs 1–3 weeks before the onset of HHV-6 reactivation (Kondo *et al.*, 2003b). This intermediate phase of viral reactivation is different from the complete reactivation phase that is characterized by the expression of productive-phase IE1/IE2 transcripts (Hummel *et al.*, 2001; Soderberg-Naucler *et al.*, 2001), and this intermediate phase seems to be relatively stable (Kondo *et al.*, 2003b).

Transfection of an IE1-expression vector into latent macrophages stimulates HHV-6 reactivation. As described above, H6LTs have latency-associated small ORFs upstream of the IE1/IE2 ORFs (Fig. 47.1). For certain other mRNAs that have small upstream ORFs (uORFs) that restrict the translation of the downstream ORFs, regulation at the translational and mRNA level is important for release from the uORF control (Hoffman *et al.*, 2001; Nomura *et al.*, 2001). An alteration in the regulation of translation as well as the increase in H6LTs might contribute to increased IE1 protein expression and viral reactivation (Kondo *et al.*, 2003b).

Since the reactivation of HHV-6 and HHV-7 is related to graft-versus-host disease in transplant recipients (Yoshikawa *et al.*, 2002) and to drug-induced hypersensitivity syndrome, these viruses might be reactivated by a strong generalized immunological response. However, the trigger that induces viral reactivation has not been identified.

Summary

In the Roseolovirus genus, some of the latency-associated transcripts of HHV-6B have been identified. Since HHV-6B and HCMV have some similarities in their latent transcripts and latent gene regulation, latency-associated transcripts of HHV-6 and HCMV might be involved in mechanizing that are common to β-herpesviruses latency. If so, similar transcripts may be identified in HHV-6A and HHV-7. Investigation of these transcripts should increase our understanding of the molecular bases of Roseolovirus latency and reactivation, about which little is known.

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48

HHV-6A, 6B, and 7: immunobiology and host response

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Introduction

The discoveries of *Human herpesvirus 6* variant A (HHV-6A), *Human herpesvirus 6* variant B (HHV-6B) and *Human herpesvirus 7* (HHV-7) followed the development of methods for activation and long-term culture of peripheral blood T lymphocytes. Based on their shared biological properties and nucleotide sequences, HHV-6A, HHV-6B, and HHV-7 are classified as members of the roseolovirus genus of the betaherpesvirus subfamily. HHV-6A and HHV-6B are very closely related, with most of their encoded proteins sharing greater than 90% amino acid sequence identity; most HHV-7 protein sequences share 30% to 60% amino acid sequence identity with their HHV-6 counterparts (for review, see Yamanishi *et al.*, 2007 and elsewhere in this volume). Roseoloviruses share many genetic and biologic properties with the more distantly related cytomegaloviruses. All of the roseoloviruses infect T lymphocytes in vivo and in vitro, cause similar damage to infected cells, and have overlapping but distinct disease spectra.

Primary infection with HHV-6B is the major cause of roseola infantum (also known as roseola, exanthem subitum, 3-day-fever, or sixth disease), a febrile rash illness common in early childhood (for review, see Braun *et al.*, 1997; Yamanishi *et al.*, 2007 and elsewhere in this volume); primary HHV-7 infections can also cause roseola, albeit less frequently. Roseoloviruses can affect the central nervous system; patients with HHV-6 primary infection sometimes develop seizures or convulsions. HHV-6A and HHV-6B infect various types of neural cells and in some studies have been associated with demyelinating diseases, including multiple sclerosis (MS) and progressive multifocal leukoencephalopathy (for review see Caserta *et al.*, 2001; Tyler, 2003). HHV-6 associated pneumonia, encephalitis, hepatitis, and hematopoietic stem cell suppression have been observed after stem cell transplantation, more often in association with HHV-6B than HHV-6A (for review see Clark and Griffiths, 2003; Ljungman, 2002; Yoshikawa, 2003). In addition, HHV-6 and HHV-7 immune suppressive activities may also indirectly contribute to fungal, bacterial, or human cytomegalovirus (HCMV) infections (Boeckh and Nichols, 2003; Ljungman, 2002). A role for HHV-6 infections in the progression of HIV disease has been debated (Braun *et al.*, 1997; Caserta *et al.*, 2001; Clark and Griffiths, 2003). Obviously, many questions remain about the pathogenesis of these viruses, with many of these questions revolving around their immunobiology.

Much of our consideration of these viruses, and justification for studying them, is based on their roles as human pathogens. This has led many experiments to be interpreted in the context of how the results might explain a pathogenic outcome that might be considered negative from the perspective of the infected host, that is, how the virus avoids the immune system and how it creates disease. Usually overlooked is the fact that, by far, the most common outcome of infection with these viruses is not disease, but a lifelong dynamic biological interaction between the virus and host that seldom manifests as disease. Thus, while the processes by which the virus evades or otherwise delays immune responses during the early stages of infection, avoids being eliminated during latency, and causes disease are important, it is worth considering that much of the immunobiology of these viruses is likely to involve a highly regulated process of *stimulation* of host responses. This stimulation must be balanced with the immunoevasive tactics to achieve a state that has almost been idealized by the roseoloviruses: healthy infected hosts capable of lifelong transmission.

Cell tropism

In this section we discuss the effects of HHV-6A, HHV-6B, and HHV-7 on cells of the hematopoietic lineage in the

context of the immunobiology of these viruses. HHV-6A and HHV-6B have apparently broader host ranges than does HHV-7; this is likely due to their choices of cellular receptors (for review, see Yamanishi *et al.*, 2007). The cellular receptor for both HHV-6A and HHV-6B is CD46, a member of the family of regulators of complement activation, which is expressed on all nucleated cells; co- or alternative receptors remain to be identified. CD4, the marker of helper T lymphocytes, is a critical component of a receptor for HHV-7. As for HSV, CMV, and HHV-8, heparan sulfate-like molecules mediate the initial HHV-7 attachment to target cell surfaces.

In vitro

All three viruses can productively infect primary CD4+ T-lymphocytes; activation of these cells is required for efficient viral replication (Table 48.1). HHV-6A, but not HHV-6B, can also efficiently infect primary CD4−/CD8+ T-lymphocytes (Table 48.1) (Grivel *et al.*, 2003; Lusso *et al.*, 1991a; Takahashi *et al.*, 1989). T-lymphocyte cell lines are widely used for cultivating these viruses: the HSB-2 and J JHAN cell lines for HHV-6A, Molt-3 and MT-4 cells for HHV-6B, and Sup-T1 cells for HHV-7. HHV-6A can also productively infect primary NK cells (Lusso *et al.*, 1993). Both HHV-6A and HHV-6B replicate in human peripheral blood monocyte-derived immature dendritic cells, and in intrathymic T progenitor cells in SCID-Hu mice (Asada *et al.*, 1999; Gobbi *et al.*, 1999; Kakimoto *et al.*, 2002; Kondo *et al.*, 1991). Although HHV-6A can productively infect terminally and incompletely differentiated cells, the virus can enter CD34+ hematopoietic stem cells and express virally encoded genes (including *U16–17* and *U91*), but not produce viral progeny (Isomura *et al.*, 2003; Knox and Carrigan, 1992; Luppi *et al.*, 1999). HHV-6 infection of fresh monocyte/macrophages does not produce virus either, although many of these cells contain viral DNA (Clark, 2000; Kondo *et al.*, 1991); as described below, these represent latent infections (Kondo *et al.*, 2002b).

In addition to cells of the immune system, HHV-6 also replicates in many other cells (summarized in Table 48.1; for review, see Braun *et al.*, 1997; Clark, 2000). Our understanding of the cell tropism of HHV-6A and HHV-6B is complicated by the fact that in the various studies, only one or the other variant was used and end points have differed, from expression of specific virally encoded mRNAs or proteins, to detection of infectious progeny.

In vitro, HHV-7 growth is restricted to activated cord blood lymphocytes, purified T lymphocytes, and CD4+ immature SupT1 cells (for review see Black and Pellett, 1999).

In vivo

An important recent observation based on improved methods for cultivation of monocytes/macrophages was that during the acute phase of primary infection, HHV-6B load (both DNA and virus titer) is nearly one log greater in these cells than in CD4+ T-lymphocytes (Kondo *et al.*, 2002a). This extends earlier work in which HHV-6B was frequently detected in CD4+ T lymphocytes obtained both during the acute phase of primary infection and from healthy adults. During the convalescent phase of primary infection and in healthy adults, HHV-6 DNA is detected primarily in monocytes/macrophages, and phorbol ester treatment induces lytic infection; this confirms that the virus establishes latency in these cells (Kondo *et al.*, 1991, 2002b). Another possible site of HHV-6B persistence is in epithelial cells that line tonsillar crypts (Roush *et al.*, 2001).

Because cells that harbor latent HHV-6 can be induced to the lytic state upon exposure to various activating stimuli, virus activity that could contribute to pathogenesis might be found in association with diverse immune stimulation events. For example, drug hypersensitivity syndrome (DHS), or drug rash with eosinophilia and systemic symptoms, presents as fever, rash, and internal organ lesions. A number of case reports have described active HHV-6 infection during DHS, but the exact role played by the virus is not clear. Two mechanisms of virus involvment have been hypothesized. Some drugs reduce B-cells and cause hypogammaglobulinemia, which has been hypothesized to trigger HHV-6 reactivation (Kano *et al.*, 2004). Alternatively, immune responses to some drugs may activate cells harboring HHV-6, leading to virus reactivation. Cases of DHS have also been linked to EBV and CMV (for review see Wong and Shear, 2004).

Little is known of the in vivo cellular distribution of HHV-6A. It has been infrequently detected in peripheral blood mononuclear cells (PBMC) and in over 50% of lungs (Cone *et al.*, 1996), but the type of cells it inhabits is not known.

HHV-6B and HHV-7 (but not HHV-6A) antigens are frequently detectable in salivary glands (Fox *et al.*, 1990; Kempf *et al.*, 1998), but only HHV-7 has been frequently cultured from saliva (Wyatt and Frenkel, 1992).

HHV-7 has been detected in over 50% of PBMC specimens collected from healthy individuals and from patients prior to bone marrow transplantation. In addition, HHV-7 antigens have been detected in a variety of lymphoid and non-lymphoid cells. The virus may establish latency

Table 48.1. Cell Tropism of HHV-6A and HHV-6B

Cells	Progeny virus[a]	Virus tested		Infected cells (%)	References
		HHV-6A	HHV-6B		
In vitro					
astrocytes	+	+	+		(He *et al.*, 1996)
epidermal cell line	–	NT	+		(Yoshikawa *et al.*, 2003a)
endothelial cells	+	+	NT	1–38	(Caruso *et al.*, 2003; Wu & Shanley, 1998)
CD34 positive stem cells	–	NT	+		(Isomura *et al.*, 2003)
immature thymocytes	–	–	–		(Roffman and Frenkel, 1990)
mature thymocytes	+	–	+		(Roffman and Frenkel, 1990)
thymocytes + anti-CD3	NT	NT	+		(Roffman and Frenkel, 1991)
thymocytes + anti-CD3 + IL2	NT	NT	+		(Roffman and Frenkel, 1991)
CBL	–	–	+	5–77	(Black *et al.*, 1989)
CBL + PHA	+	NT	+		(Black *et al.*, 1989)
CBL + PHA + IL2	+	NT	+		(Black *et al.*, 1989)
CBMC + PHA	NT	NT	+	5–6	(Kikuta *et al.*, 1990a)
CBMC + anti-CD3 + IL2	NT	NT	+	50–90	(Kikuta *et al.*, 1990a)
CBMC + PHA	NT	NT	+	35–56	(Kikuta *et al.*, 1990a)
PBMC	NT	NT	–		(Kikuta *et al.*, 1990a)
PBMC + anti-CD3	NT	NT	+	27–70	(Kikuta *et al.*, 1990a)
PBMC + PHA	NT	NT	–		(Kikuta *et al.*, 1990a)
PBL	1	NT	+	2–15	(Black *et al.*, 1989; Frenkel *et al.*, 1990)
PBL + PHA	5	NT	+		(Frenkel *et al.*, 1990)
PBL + IL2	4	NT	+		(Frenkel *et al.*, 1990)
PBL + IL2 + PHA	5	NT	+		(Frenkel *et al.*, 1990)
CD28 positive T cells	0	NT	+		(Frenkel *et al.*, 1990)
CD28 T cells + PHA	3	NT	+		(Frenkel *et al.*, 1990)
CD28 T cells + IL2	1	NT	+		(Frenkel *et al.*, 1990)
CD28 T cells + PHA + IL2	6	NT	+		(Frenkel *et al.*, 1990)
tonsilar CD4+ T cells	+	+	+		(Grivel *et al.*, 2003)
tonsilar CD8+ T cells	+	+	+	5–20[b]	(Grivel *et al.*, 2003)
NK cells		+	NT		(Lusso *et al.*, 1993)
monocytes	–	+	+		(Burd & Carrigan 1993)
macrophages					
dendritic cells	+	+	+	2	(Asada *et al.*, 1999)
dendritic cells	+	+	+	>90	(Kakimoto *et al.*, 2002)
dendritic cells	+	+	+	30–95	(Hirata *et al.*, 2001)
JJHAN cells	+	+	–	>85	(Wyatt *et al.*, 1990)
HSB-2	+	+	–	28–90	(Ablashi *et al.*, 1991; Wyatt *et al.*, 1990)
Sup T1	+	+	–	>90	(Ablashi *et al.*, 1991)
Molt-3	+	–	+	>90	(Ablashi *et al.*, 1991)
MT-4	+	NT	+		(Asada *et al.*, 1989; Black *et al.*, 1989)
MRC-5 fibroblast	+	+	–		(Robert *et al.*, 1996)
liver cell line	+	+	+	50–90[c]	(Cermelli *et al.*, 1996)
mink lung epithelial cells	+	NT	+		(Simmons *et al.*, 1992)

(*cont.*)

Table 48.1. (*cont.*)

Cells	Progeny virus[a]	Virus tested HHV-6A	HHV-6B	Infected cells (%)	References
In vivo (ES patients)					
ES patients					
CD4+ T lymphocytes	+	–	+		(Kondo *et al.*, 2002a; Takahashi *et al.*, 1989; Yasukawa *et al.*, 1998)
CD8+ T lymphocytes	–	–	+		(Yasukawa *et al.*, 1998)
Monocytes/macrophage	+	–	+	0.03–2	(Kondo *et al.*, 2002a)
SCID-hu Thy/Liv mice					
CD4+ T cell progenitors	–	+	+		(Gobbi *et al.*, 1999)
CD3+ cells	+	+	+		(Gobbi *et al.*, 1999)

CBL (umbilical cord blood lymphocytes), CBMC (umbilical cord blood mononuclear cells),
PBL (peripheral blood lymphocytes), PBMC (peripheral blood mononuclear cells), NT (not tested)
[a] $\log_{10}$ virus titer per ml of culture supernatant.
[b] 20% of tonsilar CD8+ T cells were infected with HHV-6A compared with 5% with HHV-6B viruses.
[c] 90% cells of a liver cell line were infected with HHV-6A, compared with 50% with HHV-6B viruses.

in CD4+ T-lymphocytes in vivo since it can be activated from PBMC by PHA or anti-CD3 stimulation.

Antigens

Roseolovirus genomes encode about 90 genes. The immunogenicity of individual viral proteins depends on their level of expression, novelty to the host, some aspects of their structure and/or composition, and degree of exposure to the immune system. In addition, adaptive immune effects can target different stages of the viral life cycle. For example, proteins expressed on the virion surface during lytic infection can be targets for neutralizing antibodies, and proteins needed intracellularly for virus replication can be targets of cytotoxic T-lymphocytes (CTL). Important questions relate to what antigens are presented to the immune system during latency, and how this contributes to regulation of latency and reactivation.

HHV-6A and HHV-6B antigens

Latent antigens

Latent transcription has been studied in greatest detail for HHV-6B. Kondo and co-workers identified four species of HHV-6B latency-associated transcripts (H6LTs) that are expressed from the ie1/ie2 region (Kondo *et al.*, 2002b). It remains to be determined whether the proteins encoded by these latent transcript open reading frames are expressed as protein and whether any portion of the immune response is directed at them. The HHV-6 latency transcripts are similar in structure to some CMV latency-associated transcripts that originate from both DNA strands in the ie1/ie2 region (Kondo *et al.*, 1996) and express proteins recognized by sera from healthy CMV seropositive individuals (Landini *et al.*, 2000).

Di Luca and colleagues have identified transcripts from *U94*, the HHV-6 homologue of adeno-associated virus type 2 rep gene, as the only HHV-6 transcripts detected in PBMC from healthy individuals (Rotola *et al.*, 1998). HHV-6 viral replication was blocked in cells transfected with plasmids encoding U94, further supporting a potential role for this gene during latency. Levels of anti-U94 antibodies in serum from healthy individuals are very low (Caselli *et al.*, 2002; Rapp *et al.*, 2000), but their prevalence and titers are higher in patients with MS (Caselli *et al.*, 2002).

Lytic antigens

Most of the virally encoded proteins are expressed during the lytic cycle of viral replication. Lytic antigens present in purified virion particles or infected cell lysates have been analyzed by immunoprecipitation and/or immunoblot analyses with serum from immunized mice or rabbits, or from infected humans.

HHV-6B virions are composed of more than 29 polypeptides that range in size from 30 to 280 kDa, of which about 25–30 peptides, including 8 glycoproteins, are recognized by positive human sera (Balachandran *et al.*, 1989; Shiraki *et al.*, 1989). Human immune responses to HHV-6B IE antigens were detected by immunofluorescence, although the

Table 48.2. HHV-6 MAbs

Target Protein	Antibody designation	Cross-reactivity			Neutralization	References	Commercial source
		HHV-6A	HHV-6B	HHV-7			
U86 (IE1)	AIE1-32	+	NT	NT	NT	(Mori *et al.*, 2003b)	
U91-86 (IE-2)	P6H8	+	–	NT	NT	(Arsenault *et al.*, 2003)	
U17-16 (IE-B)	B701	+	NT	NT	NT	(Flebbe-Rehwaldt *et al.*, 2000)	
U27, p41	9A5D12	+	+	±	–	(Balachandran *et al.*, 1989; Berneman *et al.*, 1992)	
U27, p41/38	C-5	+	–	±	NT	(Berneman *et al.*, 1992; Iyengar *et al.*, 1991)	Chemicon
U94 (54 kDa)	anti-REP	–	+	NT	NT	(Dhepakson *et al.*, 2002)	
U39 (gB)	6A5H7[a]	+	+	–	NT	(Balachandran *et al.*, 1989; Berneman *et al.*, 1992)	ABI
	OHV1	–	+	NT	–	(Okuno *et al.*, 1992; Takeda *et al.*, 1996)	
	2D9	+	–	–	NT	(Campadelli-Fiume *et al.*, 1993)	
	87-y-13	+	–	NT	+	(Takeda *et al.*, 1996)	
	2D10[a]	+	+	–	+[b]	(Foa-Tomasi *et al.*, 1992)	
U48 (gH)	7A2	+	+	–	–	(Anderson & Gompels 1999; Balachandran *et al.*, 1989)	
	OHV3/9	–	+	NT	+	(Okuno *et al.*, 1990; Takeda *et al.*, 1997)	
	2E4	+	+	–	+	(Foa-Tomasi *et al.*, 1991; Foa-Tomasi *et al.*, 1994; Liu *et al.*, 1993a)	
	1D3	+	+	±	+	(Liu *et al.*, 1993a; Neipel *et al.*, 1992)	
	5E7	+	+	–	+	(Liu *et al* 1993a; Neipel *et al.*, 1992)	
U100 (gQ)	2D6[a]	+	–	–	+	(Balachandran *et al.*, 1989; Qian *et al.*, 1993)	
	au100–119	+	NT	NT	NT	(Mori *et al.*, 2003a)	
	au100–124	+	NT	NT	NT	(Mori *et al.*, 2003a)	
U11 (p101)	C3108–103	–	+	NT	NT	(Pellett *et al.*, 1993)	Chemicon

NT (not tested)

[a]Several other MAbs to the same protein were described simultaneously, but the relationships among them are not clear.

[b]Neutralization only in the presence of complement.

Many other HHV-6 MAbs have also been produced, but their viral protein targets are not as well characterized.

individual protein targets were not identified (Eizuru and Minamishima, 1992).

Monoclonal antibodies (MAbs)

HHV-6-specific MAbs have been used to identify specific virally-encoded proteins and their functions (Table 48.2). Some of the MAb are variant specific, while others recognize both variants; a few also react with HHV-7 antigens.

Targets of complement independent neutralization

Anti-gB antibodies neutralize HHV-6A and HHV-6B infection and cell fusion (Mori *et al.*, 2002; Santoro *et al.*, 1999; Takeda *et al.*, 1996). The epitope of one neutralizing gB MAb (87-Y-13) is in the vicinity of amino acid 134 (Takeda *et al.*, 1996).

As for other herpesviruses, HHV-6A gH and gL depend on each other for proper processing and expression on the cell surface or viral envelopes. The epitope for the OHV3 MAb to HHV-6B gH maps near amino acid 389 (Takeda *et al.*, 1997). MAb 2E4, 5E7 and 1D3, react with gH from both HHV-6 variants via conformational epitopes that include sequences between amino acids 145 to 230, plus residue 652 (Anderson *et al.*, 1996; Anderson and Gompels, 1999). A complex of gH, gL, and gQ appears to be required for binding to the

Table 48.3. HHV-7 MAbs

Reference	Target Protein	MAb Designation	Cross-reactivity[d]	Commercial source
(Foa-Tomasi *et al.*, 1994)	U14, pp85	3B1[a]	± HHV-6A and -6B	Chemicon
(Takeda *et al.*, 2000)	U14	24G7		
	U27	5H4	+ HHV-6B	
(Skrincosky *et al.*, 2001)	gQ (U100)	19F8[a]		
(Foa-Tomasi *et al.*, 1994)	p121	3H12		
	p51	2C1		
(Nakagawa *et al.*, 1997)	gp78, 110	5E12[b]		
	gp85, 80, 45	5F12[b]		
	52 kDa	16B4		
	40 kDa	7C10[c]	++ HHV-6A and -6B	
	40 kDa	10F1[c]		
	120, 210, 180	5A3	+ HHV-6A and -6B	
	34	2B8		
(Tsukazaki *et al.*, 1998)	125 kDa	IK3[a]		
	p120	IK16		
	p85	IK5[a]		
	50 kDa	IK27		
	125 kDa	IK10	± HHV-6A and -6B	
Okuno T., personal	IFA	KR3		
communication	IFA	KR4		

[a]Two or more MAbs were made for the same protein.
[b]Neutralizating antibody
[c]MAbs recognize two different proteins.
[d]Cross-reactivity: positive by IFA and immune precipitation (++), positive by IFA (+), and weakly positve by IFA (±).
None of these MAbs cross-react with HCMV.

HHV-6 cellular receptor, CD46, since neither the gH-gL complex nor gQ expressed alone binds to CD46 (Mori *et al.*, 2003b; Santoro *et al.*, 2003). MAbs against gH neutralize viral infectivity and block formation of polykaryocytes (Balachandran *et al.*, 1989; Foa-Tomasi *et al.*, 1991; Qian *et al.*, 1993).

Targets for diagnosis

Essentially all individuals positive by immunofluorescence for HHV-6 mount a readily detectable serologic response to the U11 protein, which can be used as a marker of HHV-6 infection with respect to the other human herpesviruses, including HHV-7 (Black *et al.*, 1996b; Neipel *et al.*, 1992; Yamamoto *et al.*, 1990). HHV-6B *U11* (homologous to HCMV *UL32*/pp150) encodes a tegument protein of 101 kDa, which shares 81% amino acid identity with its HHV-6A counterpart (p100) (Neipel *et al.*, 1992; Pellett *et al.*, 1993). U11 antigenic epitopes recognized by human serum have been mapped to near its carboxyl-terminal region, which has only limited sequence similarity and no cross-reactivity with its HCMV counterpart (Neipel *et al.*, 1992). MAb C3108–103 against U11 is HHV-6B specific; the epitope is in the vicinity of amino acid 723 (Pellett *et al.*, 1993).

75% of HHV-6 positive sera reacted with the gH-gL complex expressed in transfected T-lymphocytes, but use of these glycoproteins for diagnosis has not been established (Liu *et al.*, 1993b). An ELISA based on a 110 kDa glycoprotein affinity-purified with monoclonal antibody 2E2 was reactive with 56% to 96% of human sera from different serum donor categories (Iyengar *et al.*, 1991); it is not known which HHV-6 glycoprotein this corresponds to. HHV-6 positive human sera that had high titer by IFA reacted very weakly with bacterially expressed HHV-6 major capsid protein (UL57) in immunoblots (Littler *et al.*, 1990).

Non-structural proteins are also targets of the humoral response. About 10%–30% of human sera react with HHV-6 p41, the DNA polymerase processivity factor encoded by gene *U27*; higher rates of both IgG and IgM reactivity to p41 have been observed in some patient populations (Patnaik *et al.*, 1995; Soldan *et al.*, 1997), but the basis for this is not understood. As mentioned above, responses to undefined HHV-6B IE antigens have been detected by immunofluorescence (Eizuru and Minamishima, 1992).

HHV-7 antigens

About 20 polypeptides were identified in HHV-7 infected cell lysates, seven of which were glycoproteins (Foa-Tomasi *et al.*, 1994; Nakagawa *et al.*, 1997). Two prominent HHV-7 antigens are encoded by the *U11* and *U14* genes, which encode the tegument proteins, pp89 and pp85, respectively (Stefan *et al.*, 1999).

HHV-7 monoclonal antibodies

Table 48.3 summarizes HHV-7 MAbs that have been generated in several laboratories.

Targets of complement independent neutralization

HHV-7 infection can be neutralized by MAb 5E12 and 5F12, which react with glycoproteins of 78 kDa and 85 kDa, respectively (Nakagawa *et al.*, 1997). HHV-7 gB (U39) and gQ (U100/gp65) bind to heparan sulfate-like molecules on cell surfaces, consistent with a role in viral entry; anti-gp65 antibodies block HHV-7 infectivity (Skrinkosky *et al.*, 2000).

Targets for diagnosis

Although serologic reactivity to intact HHV-7 gB is generally weak, an ELISA based on amino acids 129–152 of gB was both sensitive and specific (Franti *et al.*, 2002). U11/pp89 and U14/pp85 are sensitive and specific serologic markers for HHV-7 (Foa-Tomas: *et al.*, 1994; Stefan *et al.*, 1999), with pp85 being recognized somewhat more frequently than pp89. MAb 5E1, which recognizes U14 (pp85) is a useful marker for HHV-7 since it does not cross-react with HHV-6 antigens and can be used on formalin-fixed, paraffin-embedded tissue (Foa-Tomas: *et al.*, 1996; Kempf *et al.*, 1997).

Immunologic cross-reactivity

The most commonly used serologic methods (e.g., IFA and ELISA) cannot reliably differentiate HHV-6A from HHV-6B infections. There is limited antigenic cross-reactivity between the HHV-6 variants and HHV-7; under the conditions of most assays, this cross-reactivity can occasionally lead to false-positive determinations of an individual's serostatus.

HHV-6A and HHV-6B

A major criterion for defining and distinguishing the HHV-6 variants is their patterns of reactivity with panels of variant-specific MAbs (Table 48.2). Most MAbs generated against one HHV-6 variant react with the other, but some are variant-specific. Similarly, more than 90% of HHV-6-specific CD4+ T lymphocyte clones react with both HHV-6A and HHV-6B (Yasukawa *et al.*, 1993) (the target antigens have not been identified). This is consistent with the extensive amino acid sequence conservation between the viruses (Dominguez *et al.*, 1999). By IFA, 95% of 234 healthy adult Malaysians had similar IgG titers to HHV-6A and HHV-6B (Yadav *et al.*, 1991), as did 80% of 136 healthy adults in the United States (Chandran *et al.*, 1992). The titer differences in some individuals may reflect different responses to unique antigens of either virus, differential responses to common antigens of both viruses, or the nature of primary infection or reactivation. A reliable variant-specific serologic test is needed to better understand the biology of the two variants.

HHV-6 and HHV-7

There is some antigenic cross-reactivity between HHV-6 and HHV-7. Hyperimmune mouse sera against one virus reacts to the other (Black and Pellett, 1999). Adsorption of serum with either HHV-6A or HHV-6B antigens removes about 20–40% of HHV-7 IgG reactivity. Similarly, about 30% of CD4+ T lymphocyte clones that react with both HHV-6A and HHV-6B react with HHV-7 (Yasukawa, *et al.*, 1993), consistent with the level of their amino acid sequence conservation (Dominguez *et al.*, 1999). Some weak or uncertain cross-reactions have also been observed for some MAbs against HHV-6 or HHV-7 (Tables 48.2 and 48.3).

This limited cross-reactivity does not generally lead to HHV-6 seroconversion in individuals naïve for HHV-6 at the time of their primary HHV-7 infection. Anti-HHV-6 IgG titers increased in about 60% of individuals with prior HHV-6 infection, suggesting possible reactivation of HHV-6 by HHV-7, and/or restimulation of reactivity with shared antigens (Caserta *et al.*, 1998; Hall *et al.*, 1994; Huang *et al.*, 1997; Tanaka *et al.*, 1994; Torigoe *et al.*, 1995). Similarly, primary HHV-6 infection does not lead to HHV-7 seroconversion, and HHV-7 primary infection can occur in the presence of high titers of HHV-6 antibodies (Hidaka *et al.*, 1994; Wyatt *et al.*, 1991). However, in HHV-6 naïve individuals, primary HHV-7 infection can transiently induce cross-reactive HHV-6 IgM (213) (Fig. 48.1). In one of two patients who had HHV-7 infections that preceded HHV-6 infection, HHV-7 IgG levels rose slightly after HHV-6 infection (Yoshida *et al.*, 2002b). Simultaneous high-titer increases in HHV-6 and HHV-7 antibody titers in young children are most likely due to dual primary infection rather than cross-reactivity (Ward *et al.*, 2001). Ward and colleagues provide evidence that IFA titers above 32 can be accepted as true positives (Ward *et al.*, 2002).

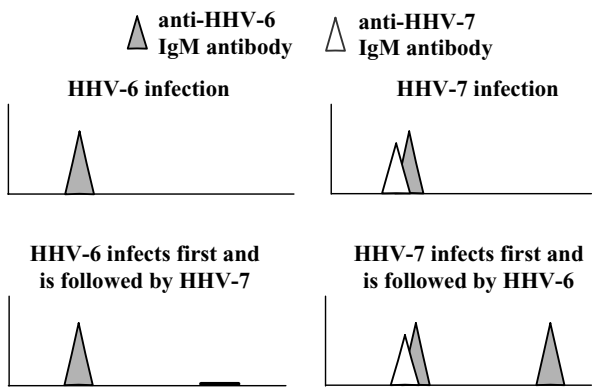

Fig. 48.1. Summary of the IgM cross-reactive responses between HHV-6 and HHV-7. From Yoshida *et al.* (2002a), courtesy of Dr. Mariko Yoshida, with permission from the American Society of Microbiology.

Roseoloviruses and other viruses

About 4% of HHV-6 specific CD4+ T lymphocyte clones responded to HCMV (Yasukawa *et al.*, 1993). HCMV vaccination or natural infection led to 4-fold or greater titer rises to HHV-6, with most of the cross reactivity in this assay being traced to the conserved gB proteins of the two viruses (Adler *et al.*, 1993). Primary infections with HHV-6 or HHV-7 can be clinically misdiagnosed as measles or rubella in children aged younger than 5 years (Black *et al.*, 1996a; Oliveira *et al.*, 2003). To complicate the matter, confirmed infections of measles, HCMV, or EBV have been associated with induction of HHV-6 reactive IgM and significant rises in HHV-6 specific IgG levels, possibly due to reactivation of HHV-6 by the other virus or a more general non-specific immune activation (LaCroix *et al.*, 2000; Linde *et al.*, 1988; Suga *et al.*, 1992a). The cross-reactivity between HCMV and HHV-7 does not confound the results of most serology assays (Black *et al.*, 1996b).

Immune response

In spite of the clear ability of these viruses to infect and kill a variety of immune effector cells both in vitro and in vivo, and to trigger a variety of mechanisms that can be seen as immunoevasive (described below), an immune response sufficient to control the infection develops rapidly following primary HHV-6 and HHV-7 infections. Thus, the likely transient and localized immune evasion or immune manipulation tactics are sufficient to allow establishment of the viral infection (including latency), but in the end have no discernable adverse long-term effects in otherwise healthy hosts. In this section, we describe immunologic outcomes such as antibody titers. In the following section we discuss regulation of these responses.

Immune response after primary infection

Innate immune response

HHV-6 and HHV-7 infections induce production of immune-regulatory chemokines and cytokines (Table 48.4). Among them, regulated upon activation, normal T expressed and secreted (RANTES), a proinflammatory β-chemokine, is known for inducing local responses by selectively attracting monocytes (the target for HHV-6 latency) and lymphocytes (a vehicle for virus spread). Interleukin-1β (IL-1β) enhances inflammatory and interferon-γ (IFN-γ) responses, and is needed for IL-6-dependent, B-lymphocyte immune responses. Both IFN-α and IFN-β inhibit HHV-6 replication in vitro (Takahashi *et al.*, 1992). However, tumor necrosis factor-α (TNF-α), normally an antiviral cytokine, may enhance release of extracellular HHV-6 by stimulating monocyte differentiation (Arena *et al.*, 1997).

HHV-6 and HHV-7 infections also enhance NK cell activity, possibly by inducing IL-15 production, which stimulates the proliferation of T lymphocytes and NK cells (Atedzoe *et al.*, 1997; Flamand *et al.*, 1996). NK cells are very effective in killing autologous HHV-6 infected cells, which may play a major role in controlling viral infection (Malnati *et al.*, 1993).

Antibody response

Given their high seroprevalence in adults, almost all infants have maternal antibodies against these viruses at birth. Antibody titers decline sharply from birth to a nadir at 3 to 6 months of age (Fig. 48.2). HHV-6 seroprevalence increases sharply from 6 months after birth into the second year, when the seroprevalence begins to approximate that in healthy adults; this is the period in which almost all HHV-6B primary infections occur (for review see Braun *et al.*, 1997; Yamanishi *et al.*, 2007). HHV-7 primary infection most often occurs after HHV-6, with seroprevalence not accumulating to adult levels until the early teens (Yamanishi *et al.*, 2007).

IgM antibodies

IgM antibodies develop 5 to 7 days after the onset of clinical symptoms, reach their highest titers in 2–3 weeks and disappear by 2 months post infection. At least one target of the IgM response is the 101K antigen encoded by

Table 48.4. HHV-6A infection and innate immunity

	Expression of immunomodulatory protein or gene			
Cells tested	Enhanced	Inhibited	Unchanged	References
Endothelial cells	IL-8 MCP-1 RANTES			(Caruso *et al.*, 2002; Caruso *et al.*, 2003)
PBMC	IL-1β			(Flamand *et al.*, 1991)
	IFN-1α			(Kikuta *et al.*, 1990b; Takahashi *et al.*, 1992)
	TNF-α			(Arena *et al.*, 1997)
	IL-10	IL-12 IFN-γ		(Arena *et al.*, 1999)
Tonsilar lymphoid tissue[a]	RANTES		MIP-1α MIP1-β IL-1α IL-1β	(Grivel *et al.*, 2001; Grivel *et al.*, 2003)
Tonsilar lymphoid cells			RANTES, MIP-1α MIP-1β	(Grivel *et al.*, 2001)
CD4+ lymphocytes		IL-2		(Flamand *et al.*, 1995)
SupT1	IL-18	IL-10 IL-14	IL-1 IL-8 IL-12 IFN-γ	(Mayne *et al.*, 2001)
Monocytes and lines	IL-10 IL12			(Li *et al.*, 1997)
	IL-15			(Arena *et al.*, 2000)
Macrophages		IL-12	RANTES TNF-α MIP-1β	(Smith *et al.*, 2003)
NK cells[b]	IL-15			(Atedzoe *et al.*, 1997; Flamand *et al.*, 1996)

[a]HHV-6B has a similar effect.
[b]HHV-7 has a similar effect.

HHV-6 *U11* (LaCroix *et al.*, 2000). An HHV-6-specific neutralizing IgM response develops after HHV-6 primary infection, regardless of whether it happens before or after HHV-7 infection. In contrast, while HHV-7 primary infection of HHV-6-naïve individuals induces IgM antibodies that can neutralize both HHV-6 and HHV-7, there is no IgM response to either virus when HHV-7 infection follows HHV-6 (Yoshida *et al.*, 2002b) (Fig. 48.1).

IgG antibodies

HHV-6 IgG antibodies usually appear within 10 days to two weeks after the onset of clinical symptoms, increase in avidity over time, and remain at measurable levels for many years (Braun *et al.*, 1997). The initial IgG response is of low avidity. Antibody avidity increases with time, making it a useful marker for identifying recent infections and for discriminating primary infections from reactivations (Ward *et al.*, 1993, 2001). HHV-6-specific IgG4 was detected in all bone marrow transplant recipients whose HHV-6 IgG antibody titers increased by at least 8-fold, whereas in pregnant women and in children less than three years of age, HHV-6-specific IgG4 was never detected (Carricart *et al.*, 2004). The possibility that IgG4 antibodies may be a marker for HHV-6 reactivation warrants further study.

Neutralizing antibodies

HHV-6 neutralizing antibodies are present at the time of birth and during and after the rash period of primary infection, but not during the febrile stage (Asada *et al.*, 1989; Suga *et al.*, 1990; Yoshida *et al.*, 2002b), suggesting that maternal antibodies have the capacity to block viral infection. Additional mechanisms may also contribute to the

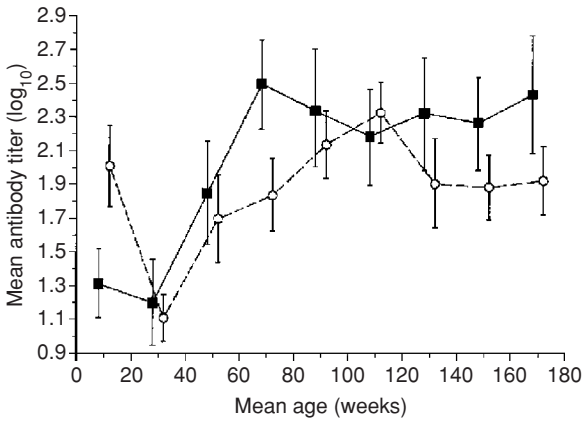

Fig. 48.2. Comparison of the change with age of the geometric mean titers of IgG antibodies to HHV-6 and HHV-7 in children. Geometric mean titers for HHV-6 (black squares) and HHV-7 (open circles) were calculated for 20-week age intervals from birth to 179 weeks. When the titer was <10 it was given a nominal value of 5. Bars represent the mean ± twice the standard error of the mean. The numbers of serum samples tested in each group were as follows: 0 to 19 weeks, 26; 20 to 39 weeks, 36; 40 to 59 weeks, 36; 60 to 79 weeks, 35; 80 to 99 weeks, 31; 100 to 119 weeks, 35; 120 to 139 weeks, 27; 140 to 159 weeks, 24; 160 to 179 weeks, 19. From Ward *et al.* (2001), courtesy of Dr. Katherine Ward, with permission from the American Society of Microbiology.

resistance to infection early in life, because antibody alone is seldom sufficient to prevent infection, as exemplified by the incomplete protection afforded by transfer of high-titered anti-CMV immunoglobulins to organ transplant recipients. Thus, in immunocompetent infants in the early months after birth, protection from HHV-6 infection and disease is the result of a combination of maternally derived antibodies (initially transplacental, then via mother's milk, some of which are neutralizing), and the child's innate immune functions.

Despite the close genetic and antigenic relationship between HHV-6B and HHV-7, their complement-independent IgG neutralizing antibodies, as detected in vitro, do not cross react with each other; this is a reflection of the differences in the viral ligands that interact with the receptors (CD46 and CD4, respectively) used by these viruses to infect target cells (Lusso *et al.*, 1994; Santoro *et al.*, 1999; Yoshida *et al.*, 2002a, b). This does not exclude the possibility that antibodies against one virus provide some protection against the others in vivo. In contrast to neutralizing IgG, HHV-7 specific IgM antibodies neutralize HHV-6 infection (Yoshida *et al.*, 2002b), but the targets have not been identified.

T lymphocyte response

HCMV nucleocapsid proteins and tegument proteins are better than the membrane glycoproteins at inducing T lymphocyte proliferation (Ljungman *et al.*, 1985). Similarly, neither HHV-6A nor HHV-6B membrane glycoproteins induce T-lymphocyte proliferation in healthy adults; instead, these antigens inhibit T-lymphocyte proliferation to mitogens or antigens (Horvat *et al.*, 1993). Antigen preparations mainly composed of the nucleocapsid proteins and tegument proteins of HHV-6A and HHV-6B induce T-lymphocyte proliferation responses of healthy seropositive children and adults (Soldan *et al.*, 2000; Yakushijin *et al.*, 1991); the response peaks later after primary HHV-6 infection (4 weeks) than for other herpesvirus infections (1 to 2 weeks) (Kumagai *et al.*, 2006). More HHV-6 seropositive, healthy adults responded to HHV-6B antigen than that to HHV-6A antigen.

As with VZV, both HLA dependent and independent cytotoxicity have been observed for HHV-6-specific CD4+ T-lymphocyte clones, suggesting heterogeneous functions of CD4+ T-lymphocytes (Arvin *et al.*, 1991; Yakushijin *et al.*, 1992).

CTLs are a product of the Th1 pathway, which is regulated in part by IL-12. HHV-6 virions are sufficient to inhibit the ability of macrophages to produce IL-12 in response to IFN-gamma or lipopolysaccharide (Smith *et al.*, 2003). Although a CTL response eventually develops, it is easy to imagine that a short deferral in the development of the response helps the infection get established.

Possible role of reactivation

About 5% of healthy adults have anti-HHV-6 IgM antibodies, suggesting periodic reactivation in the absence of clinical sequelae (Balachandra *et al.*, 1989; Ohashi *et al.*, 2002; Suga *et al.*, 1992b). During pregnancy, HHV-6 reactivation, as identified by HHV-6 DNAemia, was associated with higher IgG titers, but had no effect on IgM levels (Dahl *et al.*, 1999).

Immune responses in immunocompromised hosts

Allogeneic bone marrow and blood stem cell transplantation

HHV-6 activity is very common after blood stem cell transplantation (SCT), with HHV-6B being more commonly detected than HHV-6A. HHV-7 activity is not detected as often in PBMC, possibly due to the low CD4+ cell counts after transplantation (Boutolleau *et al.*, 2003; Miyoshi *et al.*, 2001; Wang *et al.*, 1996). Because of their high prevalences, most of the post-transplant activity of these viruses is due to either reactivation or reinfection; primary infections are

rare. It is difficult to interpret serological data in these patients because they are often severely immune suppressed and exposed to many blood products. HHV-6 antibody titers do increase (including neutralizing antibodies), but often this is not linked to the presence of viral DNAemia or infectious virus in bodily fluids (Molden *et al.*, 1997; Wilborn *et al.*, 1994; Yoshikawa *et al.*, 1991). Geometric mean antibody titers were significantly higher in recipients without HHV-6 viremia than in those with viremia (Yoshikawa *et al.*, 2002), consistent with a stronger immune response being linked to reduced virus activity. Anti-HHV-7 antibody titer increased in an allogeneic SCT patient with HHV-7-associated meningitis (Yoshikawa *et al.*, 2003b).

After SCT, reconstitution of antigen specific T-lymphocyte responses to HSV, VZV, and CMV do not correlate with the recovery of lymphocyte population levels, nor with the ability to proliferate in response to mitogens. Specific responses develop only after reactivation, reinfection, or vaccination. After allogeneic SCT, more patients showed higher levels of HHV-6B- than HHV-6A-specific T-lymphocyte proliferation responses, in agreement with HHV-6B infection being more common in this setting (Wang *et al.*, 1999). In addition, HHV-6 DNAemia was associated with a lack of HHV-6 specific T lymphocyte responses, regardless of the presence of HHV-6-associated disease, again suggesting a role of immunity in regulating viral activity.

Solid organ transplantation

As with allogeneic SCT, HHV-6B activity is common in the early stages after liver transplantation. Both reactivation and primary infection lead to HHV-6 specific IgM and IgG responses after liver transplantation, but these responses are frequently transient, the instability of the response possibly being due to a lingering immune deficit or other clinical issue (DesJardin *et al.*, 1998; Yoshikawa *et al.*, 2000). Fatal primary HHV-6A infection has been reported in the face of IgM and IgG responses after renal transplantation (Rossi *et al.*, 2001).

HHV-6 specific T-helper lymphocyte proliferation was suppressed after liver transplantation and recovered slower than for HCMV (Singh *et al.*, 2002a). Similar to patients after SCT, HHV-6 specific T lymphocyte responses are weaker in patients with HHV-6 viremia. Nonetheless, responses were normal to the T lymphocyte mitogen, Concanavalin A, suggesting a more specific deficit. Studies on transplanted livers showed that HHV-6 increases vascular expression of adhesion molecules like ICAM-1 and VCAM-1, thus inducing lymphocyte infiltration that may contribute to graft rejection (Lautenschlager *et al.*, 2002).

Multiple sclerosis (MS)

MS is considered to be an autoimmune disease of the central nervous system, in which myelin is destroyed by autoreactive T cells that target certain myelin-specific proteins. The trigger for this activity is not known, but several viruses have been suggested as potential causes or contributors, among them, HHV-6A and HHV-6B. As reviewed in detail elsewhere, experimental approaches to questions of HHV-6 involvement in MS have included (i) PCR analyses of blood, cerebrospinal fluid, and brain tissues, (ii) testing for the presence of viral antigens by immunohistochemistry, (iii) and virus-specific immune responses in blood and intrathecally. The results have been mixed and are difficult to interpret. Thus several studies have noted elevated frequencies of detecting HHV-6 infection in MS patients and in affected brains relative to other neurologic diseases or healthy controls, while others have found no differences. One tenet relating to proof of etiology is consistency of association, which certainly has not been attained with respect to observations among the several groups that have studied this problem. Nonetheless, there has been sufficient consistency of overlapping and complementary data from multiple studies from some research groups and between several groups, to preclude dismissing the possible association. Importantly, studies that have looked the closest at the affected tissues have found some of the strongest associations (Cermelli *et al.*, 2003; Goodman *et al.*, 2003). Thus, we consider this an important area for continued research.

There are several connections to immunobiology. HHV-6 is a constitutive inhabitant of brains, and MS is an inflammatory disease. It is not a stretch to imagine that the immunomodulatory activities of the virus might affect the biology of T-cells in the brain. It is also possible that unusual activity of the virus in the course of the disease might manifest itself in measurable changes in various immune markers. The virus could thus play roles in either as the direct causative agent of the disease or by modulating the course of pre-existing disease. Some recent results consistent with these possibilities are as follows.

(i) In a study that found an association between HHV-6 activity and MS activity, serum IL-12 levels were elevated during the periods of simultaneous disease and virus activity (Chapenko *et al.*, 2003). IL-12 regulates some cytokines and development of Th1 responses. Perplexing in this regard is the contemporaneously published report that HHV-6A markedly suppresses IL-12 expression in macrophages (Smith *et al.*, 2003), the discrepancy possibly being rooted in the differences between the complex in vivo situation and the in vitro purified cell population.

(ii) Cell culture supernatants from HHV-6A- and HHV-6B-infected SupT1 cells induced a non-apoptotic death in

cultured primary dendrocytes but not in primary astrocytes (Kong *et al.*, 2003).

(iii) MS patients had somewhat elevated rates of generating T cell lines that were cross-reactive between HHV-6 antigens and myelin basic protein (Cirone *et al.*, 2002). Possibly related to this, a seven amino acid segment of myelin basic protein is identical to a sequence encoded by *U24* (a protein of unknown function) of both HHV-6A and HHV-6B. This sequence is a more frequent target of cross-reactive CD4+ cytotoxic T cells in MS patients than in controls (Tejada-Simon *et al.*, 2003).

(iv) In a study in which HHV-6 DNA was detected more frequently in serum and cerebrospinal fluid of MS patients than controls, MS patients had lower frequencies of T cells that recognize the major HHV-6 antigen (101K or p100, encoded by *U11*), slightly lower IgG titers against 101K, and markedly elevated serum IgM to the same protein (Tejada-Simon *et al.*, 2002). When infected cell lysates were used as the antigens, HHV-6A specific T-lymphocyte proliferation response were detected more often in patients with relapsing-remitting MS than in the healthy controls, with no differences in the frequency of HHV-6B or HHV-7 specific responses (Soldan *et al.*, 2000). Again, these differences were not found in patients who were mainly in the chronic progressive stage (Enbom *et al.*, 1999).

(v) Results of comparisons of serologic responses to the complex array of HHV-6 antigens present in infected cells by immunofluorescence assays have shown little difference between MS cases and controls (Ablashi *et al.*, 1998, 2000; Enbom *et al.*, 1999). Interestingly, differences have consistently been seen in some studies when defined antigens (IgG to U94/rep and IgM to p41/38) have been used (Caselli *et al.*, 2002; Soldan *et al.*, 1997). IgG antibodies against HHV-6 are often detected in cerebral spinal fluids from MS patients, but it is still unclear whether this reflects real viral activity or non-specific immunoactivation (Ablashi *et al.*, 1998; Enbom *et al.*, 1997). No differences were found in HHV-6-specific IgG subclasses in sera from MS patients compared with healthy controls (Enbom *et al.*, 1999).

In sum, the accumulated diverse results suggest that in at least some MS patients, disease activity and progression are somehow connected to HHV-6 activity and immunobiology; the question remains of whether the virus contributes to the disease or is a fuzzy marker for some aspect of the disease process.

Virally mediated immune modulation

HHV-6 affects almost all the components of the immune system, including both innate and adaptive immune functions. Having discussed some of the end points of these functions, we will now discuss the underlying mechanisms. For heuristic reasons, we will trace these effects outward from the effects on infected cells, to the induction and expression of viral and cellular cytokines, and then the responses triggered by these molecules.

Cell surface markers

As summarized in Tables 48.4, 48.5, and 48.6, the roseoloviruses each trigger mechanisms that result in changes in expression of cell surface markers and other immunomodulators, leading to profound effects on immune-related cell functions and the organismal response to the infection.

Effects on specific cell types

NK cells

HHV-6 infects and kills NK cell clones cultured in vitro. In addition, HHV-6 also induces NK cells to express CD4, the helper T lymphocyte surface marker and the major cellular receptor for HIV-1, which can render NK cells permissive to HIV infection; this has led to speculation that HHV-6 not only causes immune suppression but also contributes to AIDS progression (Emery *et al.*, 1999; Lusso *et al.*, 1993). However, HHV-6 infection of PBMC, which include a variety of cell types, results in enhanced NK activity and NK-mediated killing of HHV-6 infected cells (Flamand *et al.*, 1996; Malnati *et al.*, 1993). Thus, virus-mediated killing of NK cells and NK-mediated killing of virus infected cells is regulated in part by the other cells in the system.

Stem cells

Although none of the roseoloviruses kill blood stem cells, they do affect their growth (Table 48.6). In vitro, HHV-7 inhibits the growth of granulocytic/erythroid/monocyte/megakaryocytic progenitors, but not their more differentiated progeny. In contrast, HHV-6 has less effect on the progenitors, but inhibits the growth of the more differentiated progeny (Isomura *et al.*, 1997, 2003; Knox and Carrigan, 1992; Mirandola *et al.*, 2000). Exposure of bone marrow precursors to HHV-6 inhibited their ability to respond to growth factors such as granulocyte-macrophage colony-stimulating factor and interleukin-3, and also reduced the outgrowth of macrophages from bone marrow (Burd and Carrigan, 1993). This may be due to induction of IFN-α by HHV-6 (Knox and Carrigan, 1992). HHV-6 also infects monocytes/macrophages, although these cells are not as prone to develop the typical CPE seen in CD4+ T-cells after HHV-6 infection. Nonetheless, the virus does cause dysfunction of blood monocytes and blocks their differentiation to macrophages (Burd *et al.*,

Table 48.5. Effects of HHV-6 or HHV-7 infection on cell surface markers related to immune functions

Marker	Cells	Effect	Virus tested	References
CD3	PHA stimulated lymphocytes	R	HHV-6A, -6B	(Lusso *et al.*, 1991b; Yasukawa *et al.*, 1999)
	Tonsilar cells	R	HHV-6A, -6B	(Grivel *et al.*, 2003)
CD4	PHA stimulated lymphocytes	R	HHV-7	(Lusso *et al.*, 1994; Yasukawa *et al.*, 1999)
	PHA stimulated T lymphocytes	NC	HHV-6A, -6B	(Santoro *et al.*, 1999)
	CD8+ lymphocytes	E	HHV-6A	(Lusso *et al.*, 1991a)
	γ/δ T lymphocytes	E	HHV-6A	(Lusso *et al.*, 1995)
	Tonsilar cells	E	HHV-6A, -6B	(Grivel *et al.*, 2003)
	Dendritic cells	NC	HHV-6A, -6B	(Asada *et al.*, 1999)
	NK cell	E	HHV-6A	(Lusso *et al.*, 1993)
CD46	PHA stimulated T lymphocytes	R	HHV-6A, -6B	(Santoro *et al.*, 1999)
	Tonsilar cells	R[a]	HHV-6A, -6B	(Grivel *et al.*, 2003)
	Dendritic cells	NC	HHV-6A, -6B	(Asada *et al.*, 1999)
CD80	Dendritic cells, six dpi	E	HHV-6A, -6B	(Kakimoto *et al.*, 2002)
CD83	two dpi	NC	HHV-6A, -6B	(Hirata *et al.*, 2001)
	six dpi	E	HHV-6A, -6B	(Kakimoto *et al.*, 2002)
CD86	six dpi	E	HHV-6A, -6B	(Kakimoto *et al.*, 2002)
CXCR4	PHA stimulated lymphocytes	R	HHV-6A, -6B, -7	(Cesarman and Knowles, 1999; Yasukawa *et al.*, 1999)
	Tonsilar cells	NC	HHV-6A	(Grivel *et al.*, 2001)
	Dendritic cells	NC	HHV-6A, -6B	(Asada *et al.*, 1999)
CCR5	PHA stimulated lymphocytes	NC	HHV-6A, -6B, -7	(Yasukawa *et al.*, 1999)
	Tonsilar cells	NC	HHV-6A	(Grivel *et al.*, 2001)
	Dendritic cells	NC	HHV-6A, -6B	(Asada *et al.*, 1999)
TNF receptors	J JHAN cells	E	HHV-6A, -6B	(Inoue *et al.*, 1997)
HLA-I	Dendritic cells, two dpi	R	HHV-6A	(Hirata *et al.*, 2001)
	six dpi	E	HHV-6A, -6B	(Kakimoto *et al.*, 2002)
HLA-II	two dpi	NC	HHV-6A, -6B	(Hirata *et al.*, 2001)
	six dpi	E	HHV-6A, -6B	(Kakimoto *et al.*, 2002)
	Epidermal cell line	E	HHV-6B	(Yoshikawa *et al.*, 2003a)
ICAM-1	Epidermal cell line	E	HHV-6B	(Yoshikawa *et al.*, 2003a)

R (reduced expression), E (enhanced expression), NC (no change), dpi (days post infection)
[a]The reduction is greater for HHV-6A than HHV-6B.

1993; Burd and Carrigan, 1993). There is disagreement as to whether HHV-6A or HHV-6B is the more potent inhibitor of hematopoietic stem cell growth (Carrigan, 1995; Isomura *et al.*, 1997, 2003). The mechanism for HHV-7 inhibition of blood stem cells is unknown.

T-lymphocytes

HHV-6 and HHV-7 infected T lymphocytes form balloon-like cells. These cells are usually mono- or binucleated and short-lived (Lusso *et al.*, 1991b). HHV-6A can induce cell fusion via its cellular receptor (CD46) in the absence of viral protein synthesis (Mori *et al.*, 2002; Santoro *et al.*, 1999). HHV-7 infection also induces formation of giant, multinucleated CD4+ T-cells, possibly due to polyploidization of infected cells because of interrupted cell cycles (Secchiero *et al.*, 1997, 1998a). HHV-6B infection shuts off host cell DNA synthesis, but stimulates host cell protein synthesis, which possibly also interferes with the cell cycle and creates a proper intracellular milieu for viral replication (Black *et al.*, 1992; Øster *et al.*, 2005). In SCID-Hu thymus/liver mice, HHV-6A and HHV-6B infection affects almost all of the major lymphoid cellular

Table 48.6. Effects of HHV-6 or HHV-7 infection on immune-related cell functions

Function	Cells	Effect	Virus tested	References
Growth	CD34+ stem cells	R	HHV-6B	(Isomura *et al.*, 2003)
	macrophage	R	HHV-6B	(Burd *et al.*, 1993)
Respiratory burst capacity	monocytes	R	HHV-6A, HHV-6B	(Burd and Carrigan, 1993)
Migration	PHA + lymphocytes	R	HHV-7	(Secchiero *et al.*, 1998c)
Survival				
SCID-hu Thy/Liv mice	thymocytes	R	HHV-6A, HHV-6B	(Gobbi *et al.*, 1999)
tonsilar culture	CD4+ lymphocytes	R	HHV-6A, HHV-6B	(Grivel *et al.*, 2003)
	CD8+ lymphocytes	R	HHV-6A, HHV-6B	(Grivel *et al.*, 2003)
Apoptosis				
in vitro	JJHAN cells	15%	HHV-6A, HHV-6B	(Inoue *et al.*, 1997)
	CBL + PHA	0%	HHV-6B	(Ichimi *et al.*, 1999)
	CBL + PHA + IL2	51%	HHV-6B	(Ichimi *et al.*, 1999)
in vivo (ES patients)	CD4+ T lymphocytes	31%		(Takahashi *et al.*, 1989; Yasukawa *et al.*, 1998)
	CD8+ T lymphocytes	11%		(Yasukawa *et al.*, 1998)

R (reduced)

subsets including CD4 and/or CD8 positive T-cells, but most severely depletes CD4 negative and CD8 negative intrathymic T-progenitor cells (Gobbi *et al.*, 1999). HHV-6A replicates in and kills CD4+ and CD8+ T-cells with almost equal efficiency, while HHV-6B predominantly replicates in and depletes CD4+ T-cells (Grivel *et al.*, 2003).

HHV-6A and HHV-6B induce a dramatic and generalized down modulation of the CD46 molecule in both infected and non-infected cells, while CD3 was downmodulated only in infected lymphocytes, with some differences in this activity between HHV-6A and HHV-6B (Furukawa *et al.*, 1994; Grivel *et al.*, 2003). In contrast, CD4 is upregulated after HHV-6A and HHV-6B infection, possibly via direct activation of the CD4 promoter in infected cells (Flamand *et al.*, 1998; Grivel *et al.*, 2003). HHV-6A showed a stronger effect on CD3 expression in CD4+ lymphocytes than HHV-6B, but the two viruses had similar effects on lymphoid tissues cultured ex vitro (Furukawa *et al.*, 1994; Grivel *et al.*, 2003). HHV-7 uses CD4 as its cellular receptor; CD4 but not CD3 expression is reduced after HHV-7 infection (Furukawa *et al.*, 1994; Lusso *et al.*, 1994; Secchiero *et al.*, 1998c). Down modulation of CD46 may lead to spontaneous complement activation and cytotoxicity of the affected cells. CD3 is an important component of the T-cell antigen receptor and is critical for transduction of the T-cell activation signal upon interaction with the antigen peptide-HLA complex. HHV-6 or HHV-7 infected lymphocytes lose their ability to proliferate in response to anti-CD3 antibodies and to kill virus infected cells (Furukawa *et al.*, 1994).

T-cell apoptosis

Like other herpesviruses, HHV-6 and HHV-7 lytic infections lead to host cell death via necrotic lysis (Inoue *et al.*, 1997; Secchiero *et al.*, 1997; Yasukawa *et al.*, 1998). In addition, CD4+ T-lymphocytes die from virus-triggered apoptosis. This induction is dependent on viral replication for HHV-7 and is independent of viral replication for HHV-6 (Inoue *et al.*, 1997; Secchiero *et al.*, 1997). HHV-6-mediated apoptosis is regulated by the Fas–Fas ligand system and the apoptosis induced by HHV-7 seems to be controlled by bcl-2 (Inoue *et al.*, 1997; Secchiero *et al.*, 1997). In support of this, inhibition of the apoptotic pathway by enhanced expression of bcl-2 enhances HHV-7 infection, as observed for other viruses including Epstein–Barr virus (Razvi and Welsh, 1995; Secchiero *et al.*, 1998b). Both HHV-6A and HHV-6B incorporate the important cell regulatory protein, p53, into virion particles; p53 regulates the cell cycle and protects a portion of infected cells from apoptosis (Takemoto *et al.*, 2005, Øster *et al.*, 2005). The basis for the choice between the two distinct fates (necrosis or apoptosis) of infected cells is unknown.

HHV-7 infection also induces tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) production, which provides a trans-acting signal that triggers apoptosis of nearby uninfected cells (Secchiero *et al.*, 2001). Simultaneously, TRAIL receptor 1 (TRAIL-R1) is down modulated in HHV-7 infected T lymphocytes. Thus, the virus triggers the death of nearby cells, which may protect it from the activity of immune effector cells such as NK cells and CTL, while protecting infected cells from similarly triggered death.

Proliferation

HHV-6A and HHV-6B viral envelope proteins inhibit T lymphocyte proliferation induced by phytohemagglutinin (PHA), IL-2, or antigens (Horvat *et al.*, 1993). Interaction of inactivated HHV-6A viral particles with PBMC inhibits proliferation of both CD4+ and CD8+ T lymphocytes and their responses to IL-2. This defect is apparently due to induction of defective IL-2 receptors or defects in IL-2 induced signaling pathway in these cells, as exogenous IL-2 does not correct the HHV-6 induced proliferation defect (Flamand *et al.*, 1995).

Dendritic cells

Dendritic cells are important antigen presenting cells for CD4 and CD8 T lymphocytes. Immature dendritic cells support HHV-6A or HHV-6B replication (Hirata *et al.*, 2001; Kakimoto *et al.*, 2002). Most of the infected cells are not killed, and differentiate into the mature forms, but these cells are functionally deficient and incapable of supporting lymphocyte proliferation (Kakimoto *et al.*, 2002). This is in contrast to HCMV, which selectively infects mature dendritic cells and down-regulates their cellular markers, thus impairing their function.

Histiocytes

Langerhans cell histiocytosis and hemophagocytic histiocytosis are characterized by dysregulated proliferation and migration of histiocytes (tissue macrophages). These diseases can be triggered by other diseases, use of certain drugs, or infections including HSV, HCMV and EBV. Cases of hemophagocytic histiocytosis have been associated with HHV-6 activity following organ transplantation and in previously healthy individuals (Rossi *et al.*, 2001; Tanaka *et al.*, 2002).

Cytokine production

HHV-6A, HHV-6B, and HHV-7 have profound influences on cytokine production by various immune cells (Table 48.4 and Fig. 48.3).

An important issue in the organismal response to infectious agents is the balance between the Th1 and Th2 arms of the immune system. There have been many studies of the effect of HHV-6 infection on the ability of target cells to produce cytokines that affect this balance, with sometimes diametrically opposed results. As discussed in detail by Lusso and colleagues (Smith *et al.*, 2003), this is probably due, at least in part, to the use of different cell types or cell populations in ex vivo conditions that do not fully represent in vivo regulatory circuits. IL-12 plays a pivotal role in

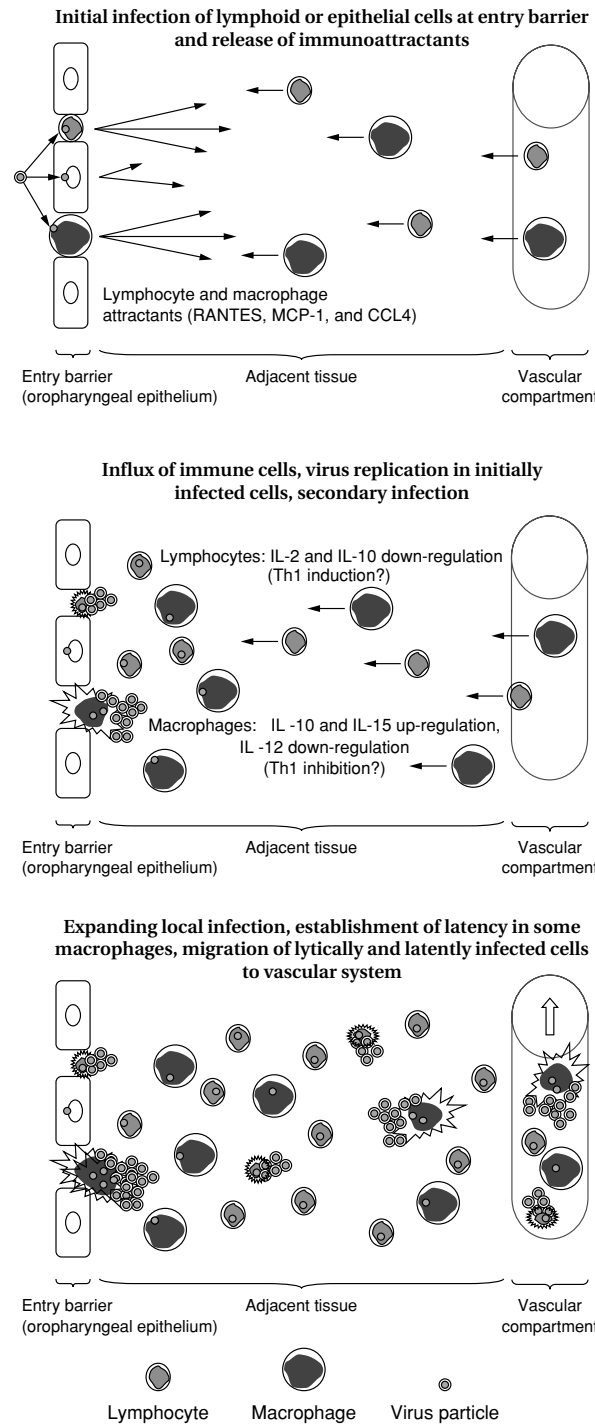

Fig. 48.3. Immunobiological events during early primary HHV-6 infection and establishment of latency. (See color plate section.)

inducing Th1 responses, and IL-10 is one of the key effectors in shifting the balance toward Th2 responses. Under some conditions, HHV-6 infection of PBMC induces IL-10 expression, which inhibits IL-12 production (Arena *et al.*, 1999; Li *et al.*, 1997). Both HHV-6A and HHV-6B infection of monocytes transiently induce low levels of IL-12 production; simultaneously, these same infections substantially restrict the level of IL-12 induction in response to IFN-γ and lipopolysaccharide; the sum is a net reduction in IL-12 production (Li *et al.*, 1997; Smith *et al.*,2003). From these results, it has been argued that HHV-6 infection might lead to inhibition of Th1-polarized immune responses. This is supported by microarray experiments involving HHV-6B infection of a T cell line that harbors HTLV-1 (Takaku *et al.*, 2005). In contrast, five days after HHV-6 infection, SupT1 cells expressed higher levels of IL-18 and other proinflammatory genes, while the level of IL-12 was unchanged and IL-10 was down-regulated (Mayne *et al.*, 2001). In addition, HHV-6 infection induces proinflammatory chemokines, including RANTES, despite encoding a beta chemokine receptor that down-regulates RANTES transcription (Caruso *et al.* 2003; Grivel *et al.*,2001, 2003; Milne *et al.*, 2000). From these data, it has been argued that HHV-6 infection leads to induction of a Th1 response (Mayne *et al.*, 2001). We have summarized these activities in a model of some of the early events following HHV-6 infection (Fig. 48.3).

Interaction of HHV-6A virions with CD4+ T-lymphocytes inhibits IL-2 mRNA synthesis and IL-2 production induced by PHA or OKT3 (Flamand *et al.*, 1995). IL-2 plays a major role in regulating T-lymphocyte and NK cell functions. HHV-6A, HHV-6B, and HHV-7 each downregulates the CXCR4 chemokine receptor leading to loss of response to the CXCR4 specific ligand, stromal cell-derived factor-1 (Hasegawa *et al.*, 2001; Secchiero *et al.*, 1998c; Yasukawa *et al.*, 1999).

Infection of primary astrocytes had only modest effects on expression of inflammatory genes. Interestingly, when the HHV-6A-infected astrocytes were treated with a pro-inflammatory mixture of TNF-α, IL-1β, and IFN-γ, anti-inflammatory genes were strongly induced (Meeuwsen *et al.*, 2005). There was very little overlap between the genes induced by infection of astrocytes vs. the HSB-2 T cell line.

Virally encoded immune modulators

Several herpesviruses, including CMV and HHV-8, encode genes homologous to human cellular chemokines or chemokine receptors, which may play important roles in pathogenesis and immune evasion. *U12* of both HHV-6 and HHV-7 encodes a functional beta-chemokine receptor (Isegawa *et al.*, 1998; Nakano *et al.*, 2003). HHV-6 *U51* encodes a functional chemokine receptor and is expressed in HHV-6 infected human endothelial cells (Caruso *et al.*, 2003; Milne *et al.*, 2000). While the product of *U12* interacts with beta-chemokines (also called CC chemokines) and activates, at least, the calcium-related signaling pathway, expression of HHV-6 U51 suppresses the transcription of RANTES, which recruits T-lymphocytes, NK cells, monocytes and eosinophils to inflammatory sites. Down-regulation of RANTES may compromise the function of these cells, as has been suggested for CMV US28. HHV-6B *U83* encodes a highly active CC chemokine that has been tested against the known chemokine receptors and interacts only with CCR2, a receptor present on macrophages and monocytes (Luttichau *et al.*, 2003; Zou *et al.*, 1999). HHV-6A U83 encodes two different forms of CC chemokines (full length being an agonist and the spliced form being an antagonist) that interact with a wide array of receptors other than CCR2 (Dewin *et al.*, 2006). These activities may contribute to the process by which the virus establishes latency in such cells.

Other immune evasion mechanisms

HHV-6 gB and gH-gL complexes are not detectable on the plasma membrane of infected lymphocytes (Cirone *et al.*, 1994), which may constitute an immune avoidance strategy. It has been reported that HHV-6A down-regulates HLA class I antigen in dendritic cells which may prevent the cells from HLA-I dependent killing, but the opposite was found in another paper (Hirata *et al.*, 2001; Kakimoto *et al.*, 2002).

HHV-7 *U21* encodes a type I membrane protein that binds to class I HLA-1 molecules and reduces their level of cell surface expression by diverting them to lysosomes (Hudson *et al.*, 2001). Unexpectedly, the redirection is not mediated by the cytoplasmic tail of U21, but by its ER-lumenal domain (Hudson *et al.*, 2003). This represents a novel mechanism for lysosomal sorting and for immune evasion.

Clinical significance of immunomodulation

A lot of interest has been concentrated on identifying the pathologic role of these viruses in immunocompromised patients. The rationale is that all the other herpesviruses cause significant diseases in this group of patients. In addition to directly causing pathologic lesions, it is also possible that these three viruses may indirectly contribute to other infectious pathogens by inhibiting the immune system (Griffiths, 2003).

Organ transplantation

HHV-6, but not HHV-7, infection is related to graft rejection in both adult and pediatric liver transplant patients (Feldstein *et al.*, 2003; Griffiths *et al.*, 1999). HHV-6 infection is related to higher CMV viral loads, CMV syndromes and diseases, bacteriemia, fungal disease, and mortality (DesJardin *et al.*, 1998, 2001; Humar *et al.*, 2000, 2002; Rogers *et al.*, 2000). HHV-6 negative patients may experience primary infection, and these patients are more likely to develop fungal infections (Dockrell *et al.*, 1999). In addition, HHV-6 may lead to hepatitis due to hepatitis C virus, and acute liver failure after liver transplantation (Harma *et al.*, 2003; Rogers *et al.*, 2000; Singh *et al.*, 2002b). Similar findings have also been made in lung and heart-lung transplant patients (Jacobs *et al.*, 2003), although HHV-6 infection was associated only with fever in one study of liver transplant recipients (217). After renal transplantation, HHV-7, but not HHV-6 DNAemia is related to graft rejection, higher plasma CMV DNA loads, CMV disease and graft dysfunction (Kidd *et al.*, 2000; Osman *et al.*, 1996; Tong *et al.*, 2000, 2002).

Prophylaxis with valacyclovir or ganciclovir, two effective anti-herpesvirus agents, lead to reduced staphylococcal, candida and other fungal infections after renal and heart transplantation (Lowance *et al.*, 1999; Wagner *et al.*, 1995). In addition to the effects such therapy may have on CMV activity, the effects of this therapy on HHV-6 and HHV-7 may have contributed to the observed benefit. Future work on therapy targeted at roseoloviruses is needed, because these viruses frequently become active earlier than CMV in many patients.

HIV disease

Because of its impact on the immune system, HHV-6 has long been hypothesized to accelerate the progress of HIV infection. HIV disease is more likely to progress in HHV-6 infected infants and active HHV-6A and HHV-6B replication is frequently observed in patients with late stage AIDS (Clark *et al.*, 1996; Emery *et al.*, 1999; Kositanont *et al.*, 1999). While this argues for either or both of the HHV-6 variants being copathogens for HIV, some data runs counter to this. Differences in methods and patient groups may account for some of the disagreement. In several studies there was no relationship between the presence of HHV-6 DNA in saliva or peripheral blood cells, or serological responses and AIDS pathogenesis (for review see Braun *et al.*, 1997).

Future directions

Several areas of roseolovirus immunobiology seem ripe for further study, including (i) events during the earliest stages of primary infection, including identification of the target cells, modulation of immune responses, and establishment of latency, (ii) interactions with innate immune functions, including triggering of innate responses by engagement of receptors such as the Toll-like receptors, (iii) further study of the targets of cellular immunity and the effectiveness of these responses during disease, (iv) the relationship between virus-mediated immune suppression and other infections, (v) the possible contributions of HHV-6 immunomodulation to MS, (vi) interactions of these viruses with the various apoptotic pathways, (vii) the circumstances under which these viruses might affect the balance between the Th1- and Th2-type responses, (viii) mechanisms for modulating HLA-1 expression or activity, and (ix) identification of the mechanisms by which the virus regulates stimulation of immunity. It will be important to extend studies that are done in cultured purified cells to systems that more fully represent the complexity of in vivo regulatory mechanisms.

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HHV-6A, 6B, and 7: persistence in the population, epidemiology and transmission

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Introduction

In common with all human herpesviruses, HHV-6 and HHV-7 establish lifelong infection following initial exposure and seroconversion. True latency as exemplified by HSV-1 and VZV, in which the genome is maintained in a transcriptionally restricted state, has not been conclusively shown for HHV-6 or HHV-7. However, the betaherpesviruses may persist in individuals via low grade replication which is continuously suppressed by a functional immune response. In this chapter we will summarize the current understanding of the epidemiology and persistence of HHV-6 and HHV-7 in the human host and its relevance to transmission. In addition, we will highlight a novel form of persistence for HHV-6 which involves integration into host chromosomal DNA.

Persistence of HHV-6 and HHV-7 in individuals

In the case of both HHV-6 and HHV-7, PCR analysis of peripheral blood mononuclear cells (PBMC) shows that a sensitive nested assay and an adequate quantity of input DNA (at least equivalent to approximately 150000 mononuclear cells or 1μg DNA) can detect viral DNA in healthy immunocompetent individuals suggestive of low levels of latent/persisting virus in peripheral blood (Jarrett *et al.*, 1990; Clark *et al.*, 1996; Kidd *et al.*, 1996). In contrast, viral loads are maintained at high levels in saliva of seropositive individuals, particularly in the case of HHV-7 (Kidd *et al.*, 1996; Fujiwara *et al.*, 2000; see Fig. 49.1). Since the salivary glands are a major site of replication following primary infection with both HHV-6 and HHV-7, saliva is likely to be an important fluid mediating transmission of virus within the community. An investigation of separate salivary glands (submandibular, parotid and lip) in multiple patients has revealed that the submandibular gland is most likely to contain HHV-6 (88%) and HHV-7 DNA (100%) compared to the other anatomic salivary glands, 50%–60% of which were infected (Sada *et al.*, 1996).

However, as described in more detail in Chapter 48 HHV-6 and HHV-7 may exist in a true latent state within cells of the lymphoreticular system and in the case of HHV-6, the central nervous system, i.e., exhibiting transcription patterns restricted to specific genomic regions without undergoing full genomic replication (Kondo *et al.*, 2002).

HHV-6 is neurotropic and analysis of postmortem brain tissue by PCR has shown that up to 85% of individuals harbor HHV-6 DNA (Chan *et al.*, 2001). In this study, HHV-6 variant B predominated in the brain although variant A was detected in 27.5% of brains and coinfections were also observed. Whether a latent transcriptionally silent or restricted expression pattern is present in this anatomic site or whether local replication can occur which facilitates viral persistence in the central nervous system, has not been determined. Following primary infection ~29% of children have HHV-6 DNA in the CSF which is consistent with the central nervous system being an important site of latency (Caserta *et al.*, 1994).

Other studies have revealed widespread distribution of HHV-6 and HHV-7 DNA in organs derived from both immunocompetent and immunocompromised hosts (Clark *et al.*, 1996; Emery *et al.*, 1999) with low viral loads detected in most tissues. However, HHV-6 viral loads were significantly increased in autopsy tissues from AIDS patients compared to controls suggesting upregulation of HHV-6 replication in the latter (Clark *et al.*, 1996).

Epidemiology of HHV-6 and HHV-7

Seroepidemiologic studies of HHV-6 and HHV-7 have used indirect immunofluoresence assays and more recently

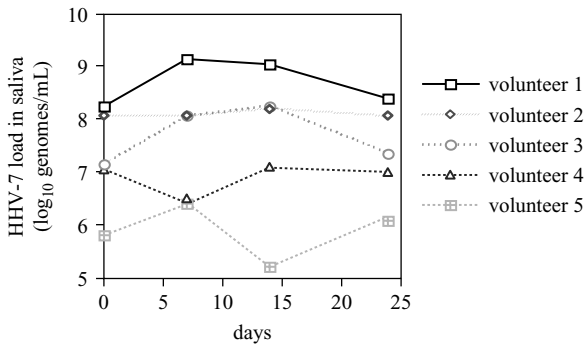

Fig. 49.1. Determination of HHV-7 loads in saliva by quantitative PCR from 5 volunteers showing temporal variation in individuals. Viral loads are expressed as HHV-7 genomes/mL saliva. Adapted from Kidd *et al.*, 1996.

ELISA based assays, with the latter deemed to be more sensitive. Antigenic similarity between HHV-6 and HHV-7 creates the potential problem of cross-reactive HHV-6 and HHV-7 antibodies in human sera (Black *et al.*, 1996). Nevertheless, many studies have consistently shown that HHV-6 and HHV-7 infections are almost universal within the adult population and the viruses have a worldwide distribution. The very close homology between the two HHV-6 variants has hindered attempts to distinguish antibodies to each variant.

Infection with both HHV-6 and HHV-7 occurs early in life. The peak of seroconversion for HHV-7 occurs after 24 months of age (Tanaka-Taya *et al.*, 1996) and is slightly later than seroconversion for HHV-6 which peaks at 13 months (Okuno *et al.*, 1989). Antibody titers are higher in children compared to adults reflecting recent primary infection. Viral loads in blood are also high during this period of acute infection and in the case of HHV-7 can reach 10^7 genomes/μg PBMC DNA. In comparison, HHV-6 loads in blood during primary infection appear to peak at around 10^5 genomes/μg PBMC DNA (Clark *et al.*, 1997; Chiu *et al.*, 1998).

HHV-6 variants A and B

HHV-6 exists as two distinct variants (HHV-6 A and B). Comparative genomic analysis of the prototypes of these variants reveals the number of highly homologous genes but also reveals significant differences in viral proteins (Gompels *et al.*, 1995; Dominguez *et al.*, 1999; Isegawa *et al.*, 1999). At present, the full implications of these differences have not been determined although it is clear that the biological phenotypes can have profound effects. The variants differ with respect to their ability to grow in a range of cell types although both use CD46 as a cellular receptor (Santoro *et al.*, 1999). Interaction with the cellular receptor has been reported to include the participation of the viral glycoproteins gH, gL and gQ (Santoro *et al.*, 2003; Mori *et al.*, 2003). gQ has only 79% amino acid identity between variants which may influence the ability of variants to target different cells. HHV-6 variant A but not variant B has also been shown to induce fusion from without in a variety of human cells mediated through the gB and gH glycoproteins and CD46 (Mori *et al.*, 2002).

A number of PCR systems that allow differentiation between HHV-6 variant A and B have been used extensively in epidemiologic studies. Some of these methods have relied on variant common primers with differentiation of variant type by differences in the presence of restriction endonuclease sites (Dewhurst *et al.*, 1992; Kidd *et al.*, 1998) or hybridization with variant specific probes (Cone *et al.*, 1996). Other studies have used variant specific primers (Chou & Marousek, 1994; Cone *et al.*, 1996).

When the prevalence of each variant is examined at different anatomical sites, HHV-6B is frequently detected in saliva (Aberle *et al.*, 1996; Tanaka-Taya *et al.*, 1996) and it is also the predominant variant in PBMC (Di Luca *et al.*, 1994) and brain tissue (Chan *et al.*, 2001). Co-infection with both variants was found in 22 of 34 lung tissue specimens (Cone *et al.*, 1996) and HHV-6A was more frequently detected in skin biopsies (Di Luca *et al.*, 1996) and CSF (Hall *et al.*, 1998). The absence of HHV-6A in clinical samples most amenable to epidemiologic investigations (blood and saliva) have prevented the true prevalence of this variant from being determined. Although HHV-6B is almost exclusively the cause of febrile illness in young children in the US (Dewhurst *et al.*, 1993), one study has suggested that active HHV-6A infection in young children with febrile illness is more common in Africa (Kasolo *et al.*, 1997).

HHV-6 and HHV-7 genotypes

HHV-6 isolates can clearly be classified as either variant A or variant B, although both are closely related with an overall nucleotide identity of 90%. There is little evidence for HHV-6 intrastrain variation although subgroups within HHV-6B have been suggested by analysis of specific regions of the viral genome (IE-A region and gH) (Chou & Marousek, 1994; Gompels *et al.*, 1993). However, examination of the U94 gene from 13 HHV-6B isolates from the US, Africa and Japan showed 100% amino acid identity (Rapp *et al.*, 2000) suggesting an intolerance of variation in this particular gene. Comparison of the two fully sequenced HHV-7 isolates (RK and JI) shows that intrastrain variation is low although

(a)

Sample time (weeks)	0	1	4	6	10	14	36	45
HHV-6 viral load (log_{10} genomes/μg PBMC DNA)	6.08	5.90	6.04	5.85	6.08	6.00	6.15	6.18

(b)

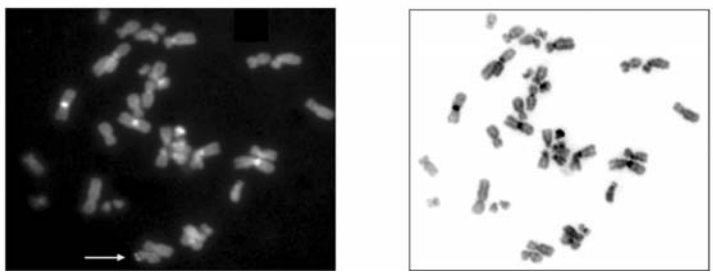

Fig. 49.2. HHV-6 integration into host cell chromosomes. (a) Consistently high viral loads detected in the peripheral blood of an individual at multiple time points by quantitative PCR (adapted from (Clark *et al.*, 1996). (b) FISH analysis using a fluorescently labeled HHV-6 specific probe showing integration of HHV-6 sequences on the short arm of chromosome 11 in V1-LCL (arrowed). (Adapted from Clark *et al.*, 2006.)

more apparent in repeat regions close to either ends of the genome (Megaw *et al.*, 1998). However, analysis of 3 genes encoding the phosphoprotein p100, gB and major capsid protein in 297 people derived from populations in Africa, Asia, Europe and America has suggested the presence of distinct variants (Franti *et al.*, 2001).

HHV-6 integration

An alternative form of HHV-6 persistence characterized by very high viral loads in PBMC and integration of viral sequences into host cell chromosomes is present in a small subset of the population. Luppi *et al.*(1993) first reported three individuals with very high viral loads in PBMC with viral DNA detectable by Southern blotting. Subsequently fluorescent in situ hybridization (FISH) with HHV-6 specific probes identified integrated viral sequences on the short arm of chromosome 17 in all three cases (B cell lymphoma, Hodgkin's disease and a patient with multiple sclerosis) (Torelli *et al.*, 1995). Follow-up studies suggested that the sites of integration were close to or in the telomeres of chromosome 17 (Morris *et al.*, 1999). Of possible relevance, HHV-6 variants have telomere-like repeat sequences in the direct repeat regions that flank the viral genome (Gompels *et al.*, 1995; Dominguez *et al.*, 1999; Isegawa *et al.*, 1999) which may facilitate integration within the telomere. The integrated viral sequences were typed as variant B in all three cases.

More recently, Daibata *et al.* (1998) described a cell line derived from a Burkitt's lymphoma patient with integrated viral sequences (variant B) on chromosome 1q44 and in a follow-up study showed that the asymptomatic husband of this case had integrated virus on chromosome 22q13 (Daibata *et al.*, 1999). Intriguingly, the daughter of this couple had integrated HHV-6 DNA sequences on both chromosomes 1q44 and 22q13 suggesting entry of viral sequences into the germline and chromosomal inheritance. The concept of an "endogenous herpesvirus" is novel and inheritance of viral sequences has only been described for endogenous retroviruses or related genetic elements. In the absence of inheritance, high viral loads found in PBMC may represent integration of viral sequences in a bone marrow progenitor cell which gives rise to a large number of cells/cell types in peripheral blood carrying copies of the viral genome. In the studies by Torelli *et al.* (1995) and Daibata *et al.* (1999), it was suggested that the entire HHV-6 genome was present.

We have also investigated HHV-6 loads in the PBMC of healthy individuals (Clark *et al.*, 1996). Using an input of 1μg DNA (equivalent to 150000 diploid cells) in a quantitative competitive PCR assay, 9 of 25 blood samples were HHV-6 DNA positive with viral loads in 8 ranging from 5–32 HHV-6 genomes/μg DNA. The remaining person (V1) had a consistent viral load of around 6 log_{10} genome copies/μg DNA in multiple samples over a ten month period (Clark *et al.*, 1996). An EBV-transformed B-lymphoblastoid cell line was established and FISH analysis using a fluorescently labeled 9 kbp HHV-6 specific probe identified integrated virus close to the end of the short arm of chromosome 11 (Fig. 49.2). The viral sequences were shown to be HHV-6B using variant typing PCR. A more extensive series of

individuals with a variety of sites of integration have now been investigated by quantitative PCR showing high viral loads are present in whole blood, serum and hair follicles (Ward *et al.*, 2006). The mean HHV-6 DNA load in whole blood was 7.0 $\log_{10}$ copies/milliliter, in serum it was 5.3 $\log_{10}$ copies/milliliter while in hair follicles it was 4.2 $\log_{10}$ copies/hair follicle.

The expression of HHV-6 genes in vivo in patients with integrated viral sequences has not been studied extensively. Daibata *et al.* (Daibata *et al.*, 1998) reported the detection of immediate early and late HHV-6 genes in an integrated HHV-6, EBV negative cell line following treatment with TPA (a commonly used stimulator of herpesvirus lytic infection). No non-integrated linear or episomal forms of the HHV-6 genome were detected by Gardella gel analysis suggesting an absence of viral DNA replication (Daibata *et al.*, 1998).

It is important to consider whether integrated HHV-6 is of clinical relevance. Although the biological effects of HHV-6 integration are unknown, integrated virus is a potential confounder in studies investigating HHV-6 disease associations and also in the medical management of infection. There have been a number of false leads and unproven associations between HHV-6 and disease, some of which may have reflected the unrecognized detection of integrated virus and interpreted as a high viral load. To date, there have been no controlled trials of antiviral therapy against HHV-6 infection, but a number of studies have shown the ability of ganciclovir and foscarnet to suppress HHV-6 replication in vitro. This information has been applied clinically to treat suspected cases of HHV-6 disease. From a management perspective, it will be important to differentiate patients with active HHV-6 infection from those patients with integrated HHV-6 to prevent the latter receiving unnecessary exposure to potentially toxic antiviral drugs.

Transmission of HHV-6 and HHV-7

Although primary infection with these viruses usually occurs in early childhood there is evidence that infection with HHV-6 occurs slightly earlier than HHV-7 (Wyatt *et al.*, 1991). Saliva is the most likely vehicle for transmission of both viruses. HHV-6 and HHV-7 DNA can frequently be detected by PCR in the saliva of both adults and children (Jarrett *et al.*, 1990; Kidd *et al.*, 1996; Suga *et al.*, 1995; Tanaka-Taya *et al.*, 1996) and although there are very few HHV-6 isolates derived from saliva, HHV-7 is readily cultured from this source (Wyatt & Frenkel, 1992). Molecular characterization of HHV-6 strains detected within a limited number of families suggest that the virus may be transmitted primarily from mother to child (Yoshikawa *et al.*, 1993; van Loon *et al.*, 1995). For HHV-7, genetic analysis of virus within families also suggests transmission within the family unit from both mothers and fathers (Thawaranantha *et al.*, 2002), and through multigenerational families in the same household (Takahashi *et al.*, 1997).

HHV-6 and HHV-7 have been detected by PCR in 20% and 3% respectively of the cervixes of women in the later stages of pregnancy (Okuno *et al.*, 1995) suggesting the potential for perinatal transmission. This observation is consistent with the identification of active HHV-6 infection during the neonatal period (Hall *et al.*, 1994). PCR analysis of cord blood suggests a prevalence of HHV-6 congential infection of 1%–2% of births (Aubin *et al.*, 1992; Adams *et al.*, 1998; Dahl *et al.*, 1999) and there are case reports of associated disease (Lanari *et al.*, 2003). HHV-6 reactivation has also been suggested to be more common during pregnancy which may facilitate transmission of virus to the fetus (Dahl *et al.*, 1999). HHV-7, but not HHV-6, was detected in 3 of 29 breast milk samples suggesting that breast feeding may also be a route of transmission for HHV-7 (Fujisaki *et al.*, 1998).

HHV-6 and HHV-7 are now recognized as being common infections in the post-transplant period. In the majority of cases, particularly adults, reactivation of the recipient's virus is the most probable source of active infection. However, transmission from the donor graft is a distinct possibility and has been reported for HHV-6 in a bone marrow transplant recipient (Lau *et al.*, 1998). HHV-6 and HHV-7 DNA have also been detected by PCR in 28% and 50%, respectively, of bone marrow samples from healthy individuals highlighting a possible source of virus for transmission through transplantation (Gautheret-Dejean *et al.*, 2000). There is also evidence suggesting transmission of HHV-6 to infants through living-related liver transplantation from their HHV-6 seropositive mothers (Yoshikawa *et al.*, 2001) and transmission of integrated virus following stem cell transplantation (Clark *et al.*, 2006).

Although HHV-6 and HHV-7 are frequently detected by PCR in PBMC including those of blood donors (Wilborn *et al.*, 1994, 1995), there is no evidence that HHV-6 at least is a cause of post-transfusion hepatitis (Lunel *et al.*, 1991).

Concluding comments

Progress is being made in understanding the persistence of HHV-6 and HHV-7 in the human host and the molecular basis for different forms of persistence. It is clear that both HHV-6 and HHV-7 are well adapted to their human

host since even in the developed world seroprevalence rates are almost universal and they are rarely pathogenic in the immunocompetent host.

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Part III

Pathogenesis, clinical disease, host response, and epidemiology: gammaherpesviruses

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50

Clinical and pathological aspects of EBV and KSHV infection

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The human γ-herpesviruses Epstein–Barr virus (EBV) and Kaposi's sarcoma herpesvirus (KSHV) establish latency in cellular reservoirs that are maintained for the life of the infected individual. Intermittent reactivation leads to infection of new cells within the host and secretion of virions in saliva. Primary infections are usually asymptomatic. However, in immunocompromised patients and in other special but poorly understood circumstances, tumors and other virus-associated diseases may manifest. Both human γ-herpesviruses were first identified in tumors and primary effusion lymphomas are typically dually infected (Cesarman *et al.*, 1995; Chang *et al.*, 1994; Epstein, 2001). For all their similarities, however, there are also striking differences between the viruses. EBV is nearly ubiquitous whereas KSHV is restricted to particular populations. EBV is most commonly associated with B-, T- and NK-cell tumors and epithelial tumors, whereas KSHV is associated with endothelial and B-cell tumors. In this chapter, aspects of virus–disease associations and therapies will be explored.

EBV

Transmission of EBV generally involves oral contact (Cohen, 2000). This might occur through the maternal chewing of food for young infants such as occurs in some cultures, the sharing of eating utensils, or kissing (Niederman *et al.*, 1976). Infection may also occur through genital transmission, blood transfusion, and organ or bone marrow transplantation (Crawford *et al.*, 2002). By adulthood more than 90% of adults show serologic evidence of EBV infection. Primary infection is usually asymptomatic in infancy or childhood, but is commonly associated with the syndrome of infectious mononucleosis when infection first occurs in adolescence or adulthood (Henke *et al.*, 1973). Symptomatic or not, infection is generally lifelong. Following initial exposure, antibody responses to some latent and lytic antigens persist indefinitely (Henle *et al.*, 1979). Thereafter, infectious virus is shed intermittently in saliva and viral DNA can be detected in peripheral blood mononuclear cells (Ling *et al.*, 2003a,b; Sitki-Green *et al.*, 2004).

Infection in the normal host

Symptomatic infection (infectious mononucleosis also referred to as glandular fever) occurs in less than half of new seroconverters during the late teenage years. The classic manifestations are fever, pharyngitis, cervical lymphadenopathy (often tender), hepatosplenomegaly, malaise and fatigue. The former symptoms and signs usually last one to three weeks, but malaise and fatigue often persist for weeks or months (Rea *et al.*, 2001).

An initial proliferation of EBV-infected B-lymphocytes is followed by the development of NK and T-cell responses (Hislop *et al.*, 2002; Tosato *et al.*, 1979) Laboratory findings include a reactive lymphocytosis (reflecting the proliferation of NK cells and T-cells), hypergammaglobulinemia and the presence of heterophile antibodies (Jenson, 2004). "Monospot" or "slide" tests are in common use and detect antigens on sheep, horse or goat erythrocytes. The appearance of heterophile antibodies appears to reflect broad spectrum B-cell activation rather than cross reaction between erythrocyte antigens and viral antigens. Heterophile antibodies typically persist for several months. Very young patients may not mount heterophile responses. In cases where the diagnosis of primary infection is in doubt, serology to assess the presence of an IgM anti-VCA response is useful. Traditionally determinations of antiviral antibodies have involved immunofluorescence, but now more commonly involve enzyme-linked immunosorbent assays.

The histologic findings in the lymph nodes range from non-specific follicular hyperplasia to a proliferation of large cells resembling lymphoma. In addition, the morphologic features may also resemble those seen in other viral infections, in particular associated with cytomegalovirus, herpes simplex or zoster, or that seen in lymph node draining sites of vaccinations. Lymph nodes from patients with infectious mononucleosis frequently have an immunoblastic proliferation of varying severity, which gives a mottled appearance to a variably expanded paracortex. Plasma cells and plasmacytoid cells are also admixed with immunoblasts and lymphocytes, so there is a polymorphous appearance. Sometimes the immunoblasts form clusters, or even sheets, partially effacing this lymph node, which may be confused with malignant lymphoma (Isaacson *et al.*, 1992).

A multitude of other manifestations are sometimes associated with primary EBV infection. These include: maculopapular rash, particularly common in patients treated with ampicillin; hepatitis; autoimmune hemolytic anemia; genital ulcers; tonsilar enlargement; aplastic anemia; and a variety of neurologic complications including encephalitis, aseptic meningitis, transverse myelitis and others (Ahronheim *et al.*, 1983). Neurologic syndromes may occur in the absence of classic signs, symptoms and laboratory findings described above (Domachowske *et al.*, 1996; Grose *et al.*, 1975; Sumaya, 1987).

In unusual circumstances, primary infection may be fatal. This may occur in the setting of congenital immunodeficiency as elaborated below or it may occur sporadically. Hemophagocytosis is often a prominent feature of fatal infectious mononucleosis (Okano and Gross, 1996).

Therapy

There is no specific therapy for infectious mononucleosis other than supportive care. Antiviral agents such as acyclovir and valacyclovir have been shown to reduce oral viral shedding but not to shorten the course of symptomatic disease (Jenson, 2004; Torre and Tambini, 1999). This lack of clinical efficacy is consistent with the idea that it is the inflammatory response to EBV rather than the destructive effects of the virus per se that are associated with symptoms. In this regard, corticosteroids which have anti-inflammatory properties and are lympholytic have been used to control pharyngitis, lymphadenopathy, splenomegaly and autoimmune manifestations such as severe thrombocytopenia or hemolytic anemia (Roy *et al.*, 2004; Tynell *et al.*, 1996).

Infection in patients with congenital immunodeficiencies

Patients with X-linked agammaglobulinemia lack B cells and seemingly lack the ability to harbor the virus (Faulkner *et al.*, 1999). Neither infectious virus nor viral DNA is detected in either saliva or peripheral blood mononuclear cells. In contrast, various forms of severe combined immunodeficiency (SCID) predispose to fatal infectious mononucleosis, hemophagocytosis, dysgammaglobulinemia and EBV-driven lymphoproliferative diseases (Elenitoba-Johnson and Jaffe, 1997; Filipovich *et al.*, 1994). Whereas SCID patients are vulnerable to many infections and are generally recognized as immunodeficient before primary EBV infection, patients with X-linked lymphoproliferative disease (XLP) often first come to medical attention in association with EBV infection (Seemayer *et al.*, 1995). The genetic defect has been mapped to the SLAM – associated protein (SAP) gene which is expressed on natural killer (NK) cells, CD4+ T-cells and CD8+ T-cells. This molecule is thought to be involved in the coordination of the immune response to EBV and other viral infections (Coffey *et al.*, 1998; Sharifi *et al.*, 2004).

Chronic active EBV

Primary infection that evolves into a severe progressive illness characterized by major organ involvement such as hepatitis, lymphadenitis and hemophagocytosis; extreme elevations of EBV antibody titers; and situations in which EBV is detected by in situ hybridization, immunohistochemistry or PCR (at high copy number) are referred to as chronic active EBV infection (Katano *et al.*, 2004). The disease is much more common in Asia with most reports coming from Japan, Taiwan, Korea and China. Mutations in the SAP gene have not been linked with chronic active EBV infection (Sumazaki *et al.*, 2001). However, in one instance chronic active EBV has been associated with a genetic defect in both alleles of the perforin gene (Katano *et al.*, 2004). Perforin is present in the granules of cytotoxic T-lymphocytes and NK cells and is required for their cytotoxic activity.

Allogeneic transplantation and EBV-specific adoptive T-cell immunotherapy have each been used alone or in combination in the treatment of chronic active EBV infection (Hagihara *et al.*, 2003; Savoldo *et al.*, 2002; Taketani *et al.*, 2002). The experience is limited, however, and reports are mixed.

Infection in HIV patients

Patients with HIV infection typically have increased antibody titers to EBV antigens, increased viral DNA copy number in peripheral blood mononuclear cells and increased viral shedding in oropharyngeal secretions (Ling *et al.*, 2003a,b; Van Baarle *et al.*, 2002). EBV is associated with a variety of malignancies in HIV-infected patients as discussed further below. It is also associated with a benign disorder, oral hairy leukoplakia (Walling *et al.*, 2003). This is a hyperplastic lesion of the oral mucosa, most commonly arising on the lateral aspect of the tongue. Occasionally these raised, white lesions occur in other immunosuppressed populations such as organ transplant recipients. Molecular analysis has revealed that several strains of virus are often present in a single lesion. The lesion is distinctive in that EBER expression is absent and there is high level expression of lytic genes (Cruchley *et al.*, 1997; Gilligan *et al.*, 1990; Webster-Cyriaque *et al.*, 2000). The condition is generally not symptomatic. However, when there are indications for treatment, lesions resolve with acyclovir or valacyclovir therapy (Walling *et al.*, 2003). Therapy is suppressive rather than curative, and recurrence following discontinuation of drug treatment is common.

Other illnesses

A variety of other chronic illnesses have been linked to EBV. In several autoimmune diseases, evidence supporting a link of some sort continues to accrue and the associations are under active investigation. These include systemic lupus erythematosus, multiple sclerosis and rheumatoid arthritis (James *et al.*, 2001; Kang *et al.*, 2004; Levin *et al.*, 2003; Yang *et al.*, 2004). Epitope spreading, cross-reactivity with autoantigens and virus-driven immortalization of autoreactive B cells have all been invoked as mechanisms whereby viral infection might precipitate autoimmunity. The possibility that autoimmunity might lead to increased EBV lytic infection in rheumatoid arthritis has also been considered (Yang *et al.*, 2004). One of the characteristic findings in rheumatoid arthritis is rheumatoid factor, an anti-immunoglobulin autoantibody. Anti-immunoglobulin antibodies lead to lytic activation in latently infected cell lines such as the Akata Burkitt's cell line. Rheumatoid factors from patients' sera have also been found to lead to lytic reactivation.

Chronic fatigue syndrome and lymphoid interstitial pneumonitis are two other illnesses that have been linked to EBV by some investigators. Chronic fatigue syndrome is a poorly understood illness in which patients have chronic fatigue and often sore throat, tender lymphadenopathy, arthralgias, memory loss or headaches (Soto and Straus, 2000; Straus, 1993). These patients often have slightly elevated antibody titers to EBV and other viruses. Lymphoid interstitial pneumonias represent a spectrum of poorly characterized diseases that include idiopathic inflammatory processes and pulmonary lymphoproliferative disorders including malignant lymphomas (Swigris *et al.*, 2002). Lymphoid interstitial pneumonia often occurs in association with other diseases, particularly Sjogren's syndrome and childhood HIV infection. EBV infected lymphocytes are often among the cells in the characteristic infiltrates, particularly in HIV-infected children but the relevance of the virus to the pathogenesis of the process is very uncertain.

EBV-associated tumors

EBV is associated with a variety of malignancies. The association between virus and tumor varies with regard to immunodeficiency, the time between primary infection and tumorigenesis, the importance of geographic factors, the importance of genetic factors, and the malignant tissue itself (lymphoid, epithelial, or smooth muscle) (Fig. 50.1).

The importance of immunodeficiency is illustrated by the observation that organ and hematopoietic stem cell transplant recipients, patients with congenital immunodeficiencies and HIV-infected patients are all at increased risk for EBV-associated B cell malignancies and the much rarer EBV-associated leiomyosarcoma (Knowles, 1999; Loren *et al.*, 2003; McClain *et al.*, 1995). In contrast, no increased risk of EBV-associated nasal lymphoma, nasopharyngeal carcinoma, or gastric carcinoma has been documented in these populations (Filipovich *et al.*, 1994; Grulich *et al.*, 2002; Hsu and Glaser, 2000; Loren *et al.*, 2003; Penn, 1996).

The time between primary infection and malignancy is typically weeks or months in organ transplant recipients, several years in Hodgkin's lymphoma patients, and several decades in nasopharyngeal carcinoma and nasal lymphoma patients (Hjalgrim *et al.*, 2003; Loren *et al.*, 2003; Raab-Traub, 2002). Environmental factors such as exposure to malaria seem to play a role in the pathogenesis of EBV-associated Burkitt's lymphoma in Africa. In regions of Africa where malaria is endemic, Burkitt's lymphoma is a common childhood neoplasm and is uniformly associated with

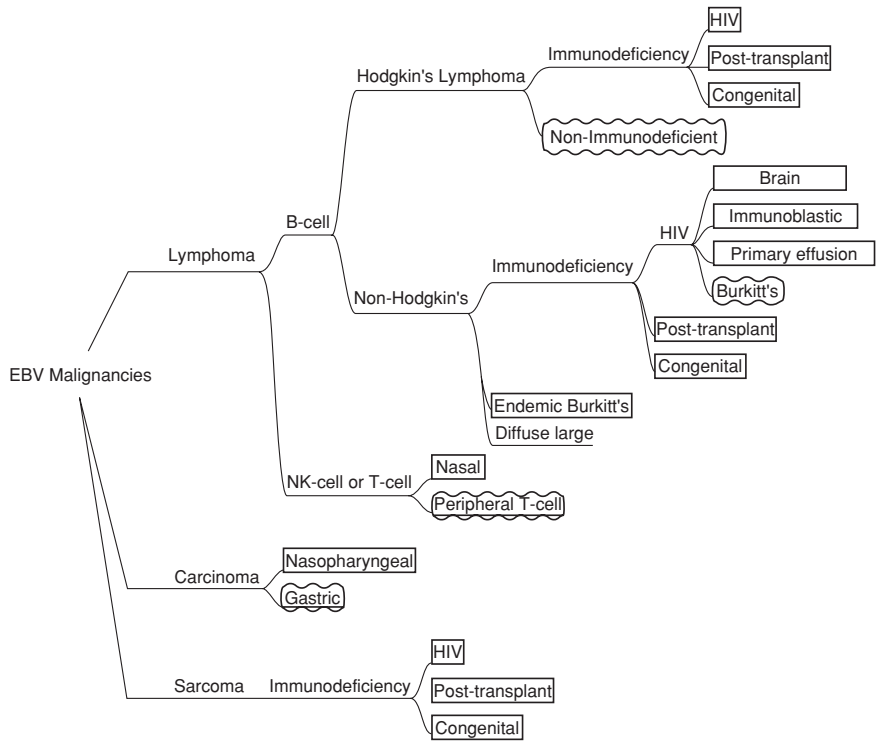

Fig. 50.1. EBV Malignancies. Rectangular boxes indicate malignancies that are almost uniformly associated with EBV. Wavy line boxes indicate malignancies that are associated with EBV in 5%–50% of cases. Blue boxes indicate an association with immunodeficiency.

EBV. In North America and Europe, Burkitt's lymphoma is much less common and is usually not associated with EBV. Populations of African descent in North America seem not to be at increased risk for the tumor (Hsu and Glaser, 2000).

A mix of environmental and genetic factors are implicated in nasopharyngeal carcinoma which is particularly common in Cantonese and Eskimo populations (Yu and Yuan, 2002). In contrast to Burkitt's lymphoma, undifferentiated nasopharyngeal carcinoma is always associated with EBV – even when it occurs in a low risk environment. When high risk Chinese populations emigrate, their risk falls but not to the Caucasian baseline. High risk families are recognized and twin studies confirm a genetic contribution (Jia *et al.*, 2004).

Finally, there is variability in the genetics of the EBV-associated tumors themselves. Burkitt's lymphomas show simple chromosomal translocations that juxtapose immunoglobulin loci, most commonly the heavy-chain locus on chromosome 14, and the c-Myc oncogene. These translocations are recognized as among the defining characteristics of the malignancy. Post transplant lymphoproliferative disorders generally lack karyotypic abnormalities. Hodgkin's lymphoma, primary effusion lymphoma and nasopharyngeal carcinoma show very complex karyotypes.

The determination of EBV association typically involves in situ hybridization for EBER RNA (abundant EBV polymerase III transcripts), or immunohistochemical detection of viral antigens (Ambinder and Mann, 1994). With the exception of peripheral T-cell lymphomas, evidence of viral infection is generally found in most of the tumor cells if any of the tumor cells harbor virus (Anagnostopoulos *et al.*, 1995). This is true at presentation and at relapse, at the primary site and in metastases. In nasopharyngeal carcinoma, several investigators have called attention to heterogeneity in the tumor, with the apparent absence of viral products from sub-populations of cells (Wu *et al.*, 1991; Yao *et al.*, 2000). With the exception of some of the lymphoid proliferations arising in profoundly immunocompromised patients, these tumors are all clonal. Clonality in lymphoid malignancies is readily established by studying antigen receptor rearrangements and in tumors in general by studying a variety of polymorphisms in X chromosomes (where in women, one or the other X will be inactivated). For the EBV-associated malignancies, it is also possible to infer clonality from the study of the viral terminal repeat sequences.

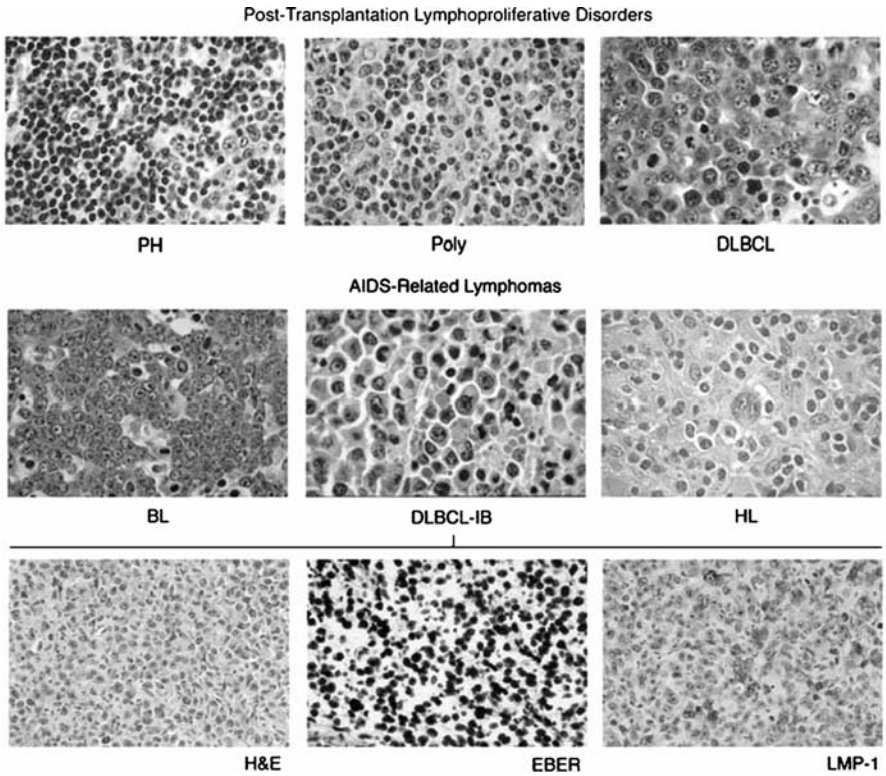

Fig. 50.2. Histopathology of EBV-associated diseases. Top row: Hematoxylin- and eosin- (H&E) stained sections of tissues involved by post-transplantation lymphoproliferative disorders are shown. These include an example of a plasmacytic hyperplasia, showing a majority of mature reactive lymphocytes with some immunoblasts and plasma cells; a polymorphic lymphoproliferative disorder, with a heterogeneous (polymorphic) cell population with atypical immunoblasts; and a diffuse large B-cell lymphoma, or monomorphic PTLD showing sheets of large neoplastic cells (original magnification 630 ×). Middle row: Examples of AIDS-related lymphomas, including: a Burkitt's lymphoma with sheets of medium-sized cells and a "starry sky" pattern due to macrophages with necrotic debris and mitotic figures; a diffuse large B-cell lymphoma with immunoblastic features (large atypical cells with an eccentric nucleus with a central prominent nucleolus and abundant cytoplasm); and an example of a Hodgkin's lymphoma with a classic Reed–Sternberg (original magnification 630 ×). Bottom row: EBV-positive AIDS-related lymphoma, H&E staining shows diffuse infiltration of neoplastic lymphocytes; in situ hybridization for EBER shows dark purple positivity in the majority of the nuclei; and immunohistochemistry for LMP-1 shows cytoplasmic positivity (brown) in many of the tumor cells (original magnification 100 ×).

Burkitt's lymphoma

This is a common childhood neoplasm in areas of Africa that are malarial (Magrath, 1997). The tumor typically arises in the jaw of young boys at the time that adult teeth are erupting. Orbital, abdominal and central nervous system involvement are common. The tumors grow exceedingly rapidly and respond to cytotoxic chemotherapy rapidly. They consist of a diffuse monotonous infiltration of B cells with many mitotic figures, as well as a high rate of spontaneous apoptosis (Fig. 50.2). A "starry sky" pattern is usually present, reflecting the presence of macrophages that have ingested apoptotic tumor cells. Immunophenotypically and genetically all morphologic forms of BL are similar: they express pan B-cell antigens such as CD19 and CD20 and are CD10 and BCL6 positive. Nearly 100% of the cells express the proliferation-associated marker Ki-67 (MIB-1).

In the endemic region, virtually all of the tumors are EBV associated. Studies suggest that antibody titers rise in anticipation of diagnosis. However, serology is not used clinically. The pattern of viral gene expression is very restricted (Niedobitek *et al.*, 1995). Most tumor cells express only latency genes. EBNA1 is expressed but not the other latency nuclear antigens or LMP1. LMP2 mRNA is detected by RT-PCR as well (Tao *et al.*, 1998). Molecular investigation of tumor tissue has shown that the promoters that drive expression of the latency nuclear antigens are densely

CpG methylated (Robertson *et al.*, 1996). In some instances, deletions of latency antigens have been documented (Kelly *et al.*, 2002). Burkitt's lymphoma cell lines are resistant to CD8 T-cell killing in vitro. This reflects their very restricted pattern of viral gene expression, a lack of surface adhesion molecules that facilitate recognition by T-cells, and a failure to process antigens for presentation in MHC class I complex (Moss *et al.*, 1999).

Although there is little doubt that malaria perturbs immune responses, it is not yet clear whether its contribution is mainly as a stimulus to B cell proliferation, as an immunosuppressive factor disrupting T-cell or NK-cell immune surveillance, or perhaps through an entirely different mechanism. Outside the endemic region of Africa, Burkitt's lymphomas are only variably (25%–80%) associated with EBV (Bacchi *et al.*, 1996). The limits of our knowledge are highlighted by the observation that in AIDS patients, the incidence of Burkitt's lymphoma is dramatically increased but these tumors occur in the least immunocompromised AIDS patients (CD4 T-cell >400) and are generally not EBV-associated (Carbone, 2003).

Post transplant lymphoproliferative disorders (PTLD)

Lymphoproliferative diseases arise in approximately 0.5 to 10% of solid organ and bone marrow or hematopoietic stem cell transplant recipients (Swinnen, 2001). The transplanted organ, the immunosuppressive regimen, and previous exposure to EBV are all determinants of risk. In organ transplant recipients tumor typically develops in host B-lymphocytes. The highest risk is associated with bowel transplantation, immunosuppression with OKT3 (a murine monoclonal antibody targeting CD3), and lack of prior exposure to EBV. In bone marrow transplant recipients, tumor typically develops in donor B-lymphocytes and the highest risk is associated with T-cell depletion of the bone marrow graft, high dose immunosuppression (again OKT3) and graft vs. host disease. The recipients' previous exposure to EBV appears to be irrelevant.

Lymphoproliferative diseases in transplant recipients have been classified as plasmacytic hyperplasia, polymorphic B-cell post-transplant lymphoproliferative disease, and monomorphic post-transplant lymphoproliferative disease (Chadburn *et al.*, 1995, 1998; Knowles *et al.*, 1995; Swerdlow, 1997). Histologically, plasmacytic hyperplasia resembles infectious mononucleosis, and is also referred to as infectious mononucleosis-like post-transplant lymphoproliferative disease. The main features are retention of the overall architecture of the tissue and presence of a mixed lymphoid population consisting primarily of lymphocytes, plasmacytoid lymphocytes, plasma cells and scattered immunoblasts; however, little or no cytologic atypia is present. The polymorphic lesions are characterized by destruction of the underlying architecture. These lesions are composed of a polymorphic (or heterogeneous) cell population, and vary from those that show extensive plasmacytic differentiation and minimal cytologic atypia to proliferations that lack plasmacytic differentiation and contain atypical immunoblasts. Individual cell necrosis to large areas of coagulative necrosis may be present. The monomorphic category includes diffuse large B cell lymphoma, Burkitt or Burkitt-like lymphoma, plasma cell myeloma, plasmacytoma and some peripheral T-cell lymphoma. In monomorphic lesions the histologic features are like those seen in immunocompetent individuals, and are classified according to standard criteria most recently described by the World Health Organization (Harris *et al.*, 1997).

EBV is usually associated with B cell lymphoproliferative disease in this setting with the exception of lesions arising several years after transplantation, which are most frequently monomorphic. T-cell lymphomas and Hodgkin's lymphoma in the post-transplant setting are also often EBV associated. As the classification implies these tumors are quite heterogeneous and many different patterns of viral antigen expression are recognized. In most series these tumors are associated almost exclusively type 1 virus (Frank *et al.*, 1995; Tao *et al.*, 2002). Some of these tumors, particularly the plasmacytic hyperplasias and polymorphic B cell post-transplant lymphoproliferative disease, express the full spectrum of antigens expressed by EBV-immortalized lymphoblastoid cell lines. They also express adhesion molecules, class I and class II molecules and are thus particularly susceptible to immune interventions.

The observation that in some cases of post-transplant lymphoma, tumor will regress if immunosuppression is withdrawn or reduced first suggested that lack of immune surveillance plays a critical role in the pathogenesis of these lesions (Porcu *et al.*, 2002; Starzl *et al.*, 1984). This idea was strengthened when it was demonstrated that adoptive cellular immunotherapy was effective in treatment and in the prevention of these tumors, particularly in the bone marrow transplant setting (Papadopoulos *et al.*, 1994; Rooney *et al.*, 1998).

Monitoring viral copy number in peripheral blood mononuclear cells, plasma or whole blood has shown that patients with post-transplant lymphoproliferative disease generally have higher copy numbers – although some patients without tumor also sustain very high viral loads (Rowe *et al.*, 2001; Yang *et al.*, 2000). Several investigators have advocated monitoring viral load as a guide to

immunosuppression or to "pre-emptive therapy" (Rowe *et al.*, 2001).

Anti-B cell antibodies have proven very useful in the management of post-transplant lymphoproliferative disease (Gruhn *et al.*, 2003; Yang *et al.*, 2000). A chimeric antibody directed against CD20, rituximab, is widely used in the treatment of B-cell lymphomas in general and is useful in the treatment of post-transplant lymphoma as well. This antibody rapidly depletes B cells in the peripheral blood and thus treatment is virtually always associated with a fall in EBV load as measured in peripheral blood mononuclear cells. However, this fall is independent of tumor response.

AIDS lymphomas

A large fraction of AIDS lymphomas are EBV associated. Brain lymphomas in AIDS patients are virtually always EBV-associated (Camilleri-Broet *et al.*, 1997; MacMahon *et al.*, 1991). Diffuse large B-cell lymphomas with immunoblastic features are also usually EBV-associated (Knowles, 1999).

Many of these tumors express the full spectrum of viral latency genes. In contrast, AIDS Burkitt's lymphoma and primary effusion lymphoma show highly restricted pattern of EBV gene expression (Horenstein *et al.*, 1997). The latter, while usually EBV associated are consistently KSHV associated and are discussed in more detail below. EBV-associated AIDS lymphomas are most common in patients with low CD4 T cell counts. In the case of primary central nervous system lymphomas, CD4 T cell counts were 10/ul in a recent cooperative group study (Ambinder *et al.*, 2003). However, it should be noted that this generalization only applies to non-Hodgkin's lymphomas in HIV patients. Hodgkin's lymphoma typically arises in HIV infected patients with CD4 T cell counts greater than 200 (Glaser *et al.*, 2003). EBV serology has not been found to be useful in identifying patients at risk for lymphoma insofar as virtually all HIV-infected patients with the exception of infants are EBV seropositive. Furthermore, EBV serology has not been helpful in the diagnosis of lymphoma in this setting. Similarly EBV copy number in peripheral blood mononuclear cells is elevated in HIV-infected patients and this elevation is not restricted to those with tumors (Van Baarle *et al.*, 2002). However, detection of viral DNA in cerebrospinal fluid is useful in the differential diagnosis of brain lymphoma in HIV patients (Antinori *et al.*, 1999; De Luca *et al.*, 1995).

Extranodal T/NK cell tumors

These lymphomas are often EBV-associated. They have been classified into nasal, intestinal, and subcutaneous panniculitis-like (Chan *et al.*, 1997; Chiang *et al.*, 1996; Jaffe *et al.*, 1999). Extranodal NK/T-cell lymphomas are characterized histologically by a diffuse lymphomatous infiltrate that is frequently angiocentric and angiodestructive. Coagulative necrosis and mucosal ulceration are common. The degree of association with EBV appears to be determined by both anatomic site and geography. Nasal lymphomas that express CD56 are most common in Asia and are almost always associated with EBV (Cheung *et al.*, 2003). EBV gene expression is similar to that of EBV-associated Hodgkin's lymphoma (EBNA1, LMP1 and LMP2 are expressed but not EBNA2, 3A, 3B, or 3C) (Chiang *et al.*, 1996). The presence of virus helps to distinguish the aggressive NK cell leukemias from more indolent NK lymphoproliferative disease (Gelb *et al.*, 1994).

Nasopharyngeal carcinoma

Most common in Cantonese, nasopharyngeal carcinoma also occurs in Arab, and Eskimo populations (Yu and Yuan, 2002). They typically present in middle age but sometimes occur in adolescents. EBV is consistently associated with the non-keratinizing and undifferentiated subtypes of nasopharyngeal carcinomas. Biopsies show lesions composed of large neoplastic epithelial cells disposed in a syncytium-like array. Abundant, normal appearing lymphocyes are admixed with the epithelial cells, giving rise to the misnomer of lymphoepithelioma. Well-differentiated tumors are often EBV associated as well (Pathmanathan *et al.*, 1995).

Nasopharyngeal carcinoma grows along nerve sheaths and invades the base of the brain and metastasize to lymphatics. Localized tumors are curable with radiation therapy. Metastatic tumors often respond to chemotherapy.

Nasopharyngeal carcinoma expresses a narrow spectrum of viral antigens with EBNA1, LMP2 and sometimes LMP1. NPC is associated with high antibody titers against many EBV antigens including lytic viral antigens (Connolly *et al.*, 2001; Milman *et al.*, 1985). The antibody profile is particularly distinctive because of the presence of IgA antibodies. Antibody titers begin arising years in advance of diagnosis and are sometimes used clinically in the evaluation of patients at high risk (perhaps because of several affected family members).

Viral DNA is present in plasma in patients with nasopharyngeal carcinoma and has emerged as a highly reliable guide to determining prognosis and monitoring therapy (Lin *et al.*, 2004; Lo *et al.*, 2000). This DNA is not encapsidated but is fragmented DNA released from tumor cells undergoing apoptosis (Chan *et al.*, 2003). Spontaneous apoptosis is an ongoing process in most rapidly growing

tumors. Radiation and chemotherapy accelerate the process. High levels of viral DNA in plasma at the start of therapy has emerged as the single most important prognostic factor, persistence of high levels in the face of therapy has emerged as a marker of relapse or progression (Chan *et al.*, 2002).

Except in Eskimo populations, these tumors are almost exclusively associated with type 1 virus (Raab-Traub, 2002). A "Chinese strain" has been identified with the number of variations, most notably in the carboxyterminus of the LMP-1 gene where a 30 bp deletion is characteristic.

Adoptive T cell therapy has been explored without much success, perhaps reflecting the very limited antigenic targets (Chua *et al.*, 2001; Moss *et al.*, 1999). There is an interesting preliminary report suggesting the possibility that peptide vaccination is efficacious (Lin *et al.*, 2002). Standard therapy involves external beam radiation often with cytotoxic chemotherapy.

Hodgkin's lymphoma

Approximately one third of Hodgkin's lymphomas are EBV-associated in North America and Western Europe, with a higher fraction in the rest of the world approaching 100% in areas of Africa and Latin America (Ambinder *et al.*, 1993; Chang *et al.*, 1993; Glaser *et al.*, 1997). The tumor usually arises in young adults (15–35 years) with the age incidence curve varying somewhat in different regions of the world. The disease most commonly presents as cervical or supraclavicular lymphadenopathy, may be associated with fevers and night sweats and can often be cured with chemotherapy, radiation therapy or combined modality therapy.

Infectious mononucleosis was recognized as a risk factor long before EBV was recognized as the cause of infectious mononucleosis (Grufferman and Delzell, 1984). A recent study has shown that infectious mononucleosis is associated with an increased incidence of EBV-associated Hodgkin's lymphoma but not other Hodgkin's lymphoma (Hjalgrim *et al.*, 2003). The typical interval between symptomatic primary infection and diagnosis of Hodgkin's lymphoma is 2 to 3 years, but increased risk continues for up to 20 years.

The diagnostic hallmark of Hodgkin's lymphoma is the presence of a minority of neoplastic cells, Reed–Sternberg cells, in a background of non-neoplastic cells. The two major subtypes of Hodgkin's lymphoma are the classical and the lymphocyte predominant forms, but only the former is associated with EBV infection. The histologic subtypes of classical HL are nodular sclerosis, mixed cellularity, lymphocyte rich classical, and lymphocyte depleted. EBV is most frequently associated with the mixed cellularity subtype, and also lymphocyte depleted, especially in the context of HIV infection. While the tumor cells are of B cell origin, B cell antigens like CD20, CD79A and immunoglobulin are frequently negative or weakly expressed. The B cell-specific activator protein (BSAP)/PAX5 can usually be detected by immunohistochemistry. EBV can be detected by in situ hybridization for EBER when present. In these cases, immunohistochemistry can detect expression of LMP-1 and EBNA-1, without EBNA-2.

Although viral antibody titers in patients with newly diagnosed Hodgkin's lymphoma differ from healthy counterparts, the differences are sufficiently small that they have no clinical utility (Chang *et al.*, 2004). Studies are ongoing to determine whether viral DNA in plasma has the same significance in Hodgkin's lymphoma that it does in nasopharyngeal carcinoma.

Gastric cancers

Carcinomas of the stomach, particularly those arising in the gastric antrum carry EBV episomes and express EBNA1 in approximately 10–15% of cases. The incidence of this malignancy varies widely with higher incidences particularly in Japan. However, the percentage of cases associated with virus seems to be relatively constant whether tumors from high or low incidence populations are being studied (Fukayama *et al.*, 1998; Takada, 2000). Although only a minority of these tumors are EBV associated, gastric carcinoma is one of the more common tumors world wide and EBV-associated gastric cancers may well be the most common EBV-associated malignancy when considered globally.

Other EBV-associated tumors

Leiomyosarcomas in the immunocompromised (organ transplant recipients, HIV-infected patients and patients with congenital immunodeficiency) are extraordinarily rare tumors but seem to be commonly EBV-associated (McClain *et al.*, 2000; Reyes *et al.*, 2002; van Gelder *et al.*, 1995). Their pattern of viral gene expression has only had limited study due to their rarity but appears to be distinctive with expression of EBNA-2 but not LMP-1. Lymphomatoid granulomatosis is an EBV-associated B cell lymphoproliferative disease that often involves skin, lungs, brain, kidney and other organs. It is associated with systemic symptoms and areas of necrosis within the tumor. The disease generally arises in older patients. As with leiomyosarcomas the disease is so rare that studies of antigen expression are quite limited. Undifferentiated thymic carcinomas resemble nasopharyngeal carcinomas histologically and are generally EBV-associated.

Breast cancer, hepatocellular cancer, thymoma and other tumors

A multitude of other tumors have been linked with EBV. Breast cancer in particular has received a great deal of attention (Bonnet *et al.*, 1999; Labrecque *et al.*, 1995). Reports suggested that viral nucleic aids and proteins could be found in a large percentage of tumors by Western blot and by in situ hybridization. However, a whole series of studies have either failed to confirm the initial reports or have offered alternate explanations such as cross-reacting antibodies or increased tendency to lytic infection in malignant tissues (Glaser *et al.*, 1998; Huang *et al.*, 2003; Murray *et al.*, 2003). Similarly, there have been reports of EBV in hepatocellular cancer, thymoma and several other malignancies that have not generally been confirmed.

The clinical importance of the viral association

Many of the tumors above are only sometimes associated with EBV, with the determinants of association varying from cofactors such as malaria (Burkitt's lymphoma) to AIDS (brain lymphoma) to other factors (Hodgkin's lymphoma). Although the detection of EBV in the tumor is sometimes useful in helping to establish a diagnosis (as in brain lymphoma in AIDS patients), there are few clinical settings in which the detection of virus per se clearly alters the prognosis or therapy. Evidence has been presented that survival in older patients may be poorer in Hodgkin's lymphoma associated with EBV than in that not associated, but definitive studies in this regard are still lacking and no one has yet advocated different primary therapy for Hodgkin's as a function of EBV status (Clarke *et al.*, 2001). For patients who have failed standard therapies, novel approaches to therapy are being explored. In some instances these approaches involve adoptive immunotherapy, in others activation of viral gene expression. Activation of viral gene expression might lead either to direct cell killing, to expression of dominant viral antigens that might render tumor cells more susceptible to immune surveillance or to sensitization to ganciclovir or similar agents whose cytotoxic activity is dependent on phosphorylation by viral kinases (Chan *et al.*, 2004; Moore *et al.*, 2001; Feng *et al.*, 2004; Ambinder *et al.*, 1996).

KSHV

Endemic in sub-Saharan Africa and in regions near the Mediterranean Sea, KSHV-seropositivity is rare in most parts of the world (Chatlynne *et al.*, 1998; Gao *et al.*, 1996). The virus is transmitted in childhood within families in endemic regions, through sexual contacts in adults in high risk groups (Kedes *et al.*, 1996; Martin *et al.*, 1998; Smith *et al.*, 1999). KSHV is commonly detected in the saliva in seropositives and salivary transmission is likely (Ablashi *et al.*, 2002; Koelle *et al.*, 1997). Some investigators have specifically implicated receptive anal intercourse, oral-genital contact, or oral-anal contact in virus transmission (Dukers *et al.*, 2000; Grulich *et al.*, 1999). Although viral DNA has been detected in seminal fluid, relatively infrequent detection in comparison with saliva and low copy number make the role of seminal fluid in transmission uncertain (Pellett *et al.*, 1999).

Primary KSHV infection

A febrile illness and maculopapular rash may be associated with primary infection in children without immunocompromise (Andreoni *et al.*, 2002). Lymphadenopathy has been associated with seroconversion in men who have sex with men (Casper *et al.*, 2002). Fever, lymphoid hyperplasia, splenomegaly and pancytopenia have been reported in organ transplant recipients (Luppi *et al.*, 2003).

KSHV-associated angioproliferative and lymphoproliferative disorders

The virus was originally discovered in Kaposi's sarcoma (Chang *et al.*, 1994). It is also present in multicentric Castleman's disease and associated plasmablastic lymphoma, and primary effusion lymphoma (PEL).

Kaposi's sarcoma

First described in older men of Mediterranean descent, Kaposi's sarcoma also occurs in regions of Africa where it afflicts children as well as adults, in immunocompromised organ transplant recipients and in HIV-infected individuals. In the latter setting, it is one of the defining illnesses of AIDS and is the most common associated malignancy.

Kaposi's sarcoma lesions often involve the skin or mucous membranes and appear as flat violaceous plaques or nodules. Lesions are generally not painful (except occasionally when they involve the plantar surface of the feet). Skin lesions often appear on the lower legs and it has been hypothesized that this reflects a predilection for relatively hypoxic regions of the body (Haque *et al.*, 2003). Although Kaposi's sarcoma lesions are often disfiguring, and associated edema may be a source of discomfort, they are rarely painful and rarely a cause of death (Fig. 50.3). Lesions commonly involve the gastrointestinal tract and the lungs. The appearance is sufficiently characteristic that

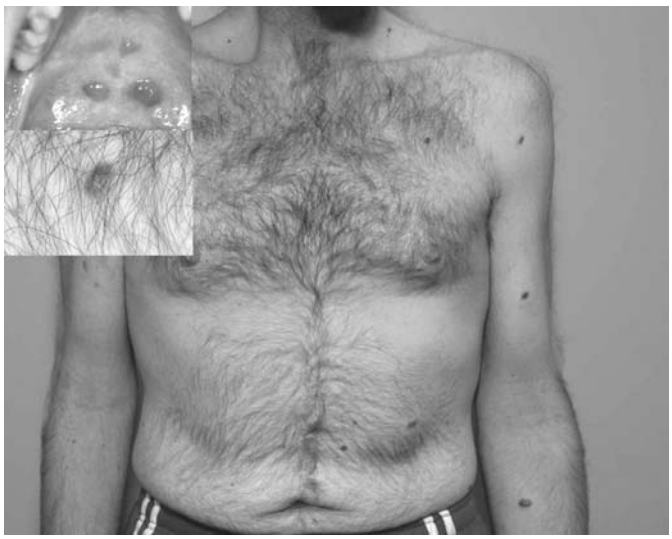

Fig. 50.3. Kaposi's sarcoma. Lesions are often scattered over the trunk and lower extremities. Their appearance is violaceous as a consequence of their neovascularity. The insets show Involvement of the hard palate (top) and a close up of a cutaneous lesion (bottom).

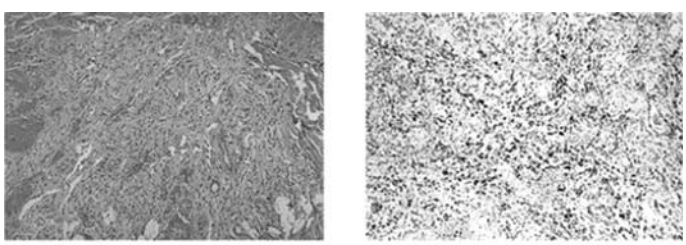

Fig. 50.4. Histological section stained with hematoxylin and eosin of a nodular tumor stage lesion of KS. Note the spindle cell proliferation and abundant vasculature. KSHV LANA (ORF-73) expression in KS. Staining with a rat monoclonal antibody revealed LANA positivity (diaminobenzidine, brown) in the nuclei of many spindle cells in a KS lesion. Positivity was also identified in endothelial cells lining the larger vascular spaces that may represent lymphatic vessels (original magnification 100 ×).

biopsy confirmation is not always necessary. Gastrointestinal lesions may be associated with hemorrhage, diarrhea, or obstruction; while pulmonary lesions may lead to fatal respiratory compromise.

The histologic characteristics of the lesions are similar across different affected populations. There are variable mixtures of ectatic, irregularly shaped, round capillary and slit-like endothelial-lined vascular spaces and spindle-shaped cells often with an inflammatory mononuclear cell infiltrate (Fig. 50.4). Red blood cells and hemosiderin pigment are frequently present, often extravasated between the spindle cells. Small granules of intracytoplasmic or extracellular hyalin material may be identified. In early lesions, the spindle cells are low in number compared to the surrounding inflammatory cells. Sometimes the earliest patch and plaque stage lesions are difficult to distinguish from granulation tissue. The spindle cells eventually become the predominant cell population, forming fascicles that compress the vascular slits, and the lesions become progressively nodular (Cockerell, 1991; Lever and Schaumburg-Lever, 1990).

Immunohistochemistry using monoclonal antibodies to LANA, shows that the viral protein is invariably expressed in Kaposi's sarcoma lesions. Nuclear staining with a speckled pattern can be seen in a variable proportion of the spindle cells, endothelial cells lining vascular spaces and in a very small proportion infiltrating CD45+/CD68+ monocytes (Katano *et al.*, 1999; Kellam *et al.*, 1999a,b; Parravicini *et al.*, 2000). Most spindle cells show expression of a restricted set of viral proteins, although a small percentage of cells express lytic antigens (Cannon *et al.*, 1999; Chiou *et al.*, 2002). It has been suggested that these lytic proteins may exert a paracrine effect on surrounding cells.

Spindle cells are thought to originate from circulating peripheral-blood hematopoietic precursor cells (Dupin *et al.*, 1999). In organ transplant recipients, tumor spindle cells may be of donor origin (Barozzi *et al.*, 2003). KS lesions are clonal in at least some instances Rabkin *et al.*, 1997; Gill *et al.*, 1998). There is the possibility that KS begins as a polyclonal inflammatory lesion that sometimes progresses to oligoclonal or monoclonal neoplasia.

Treatment

Asymptomatic lesions do not always require treatment. In AIDS patients, lesions may regress or stabilize with control of HIV (Krown, 2004). Lesions may be treated topically, with a retinoid cream, injections of cytotoxic agents, or irradiation. Disseminated and visceral disease may respond to systemic treatment with alpha interferon or cytotoxic chemotherapy. Liposomal formulations of anthracyclines have proven particularly useful. These agents which are generally not associated with either nausea or alopecia are generally very well tolerated. Paclitaxel is also very active but is often reserved for second-line treatment because of its toxicity profile with severe myalgias and nausea common. There is no established role for antiherpesviral agents such as foscarnate, ganciclovir or cidofuvir in the management of KS, although there is an intriguing report that

ganciclovir may reduce the incidence of KS in HIV patients being treated for cytomegalovirus retinitis (Robles *et al.*, 1990; Martin *et al.*, 1990).

Castleman's disease and plasmablastic lymphomas

Castleman's disease is a poorly understood atypical lymphoproliferative disorder, usually described as a polyclonal, non-neoplastic condition. Two distinct histopathologic subtypes had been reported before the identification of KSHV. The hyaline vascular type, by far more common, and the plasma cell type.

Castleman's disease can be localized or may be multicentric involving many lymph node groups and spleen. Multicentric Castleman's disease (MCD) is characterized by recurrent fevers, lymphadenopathy, hepatosplenomegaly, autoimmune phenomena and not infrequently progresses to lymphoma or Kaposi's sarcoma, consistent with an association with KSHV infection (Soulier *et al.*, 1995). MCD, also called multicentric angiofollicular hyperplasia, is characterized by a vascular proliferation in the germinal centers, which is reminiscent of KS (Fig. 50.5). In fact, the presence of a lymph node containing both KS and Castleman's disease is not uncommon in HIV-positive patients.

The histologic appearance of Castleman's disease is quite different in the hyaline vascular and plasma cell types. The former is characterized by enlarged lymphoid follicles, small hyalinized germinal centers within an expanded concentric mantle of small lymphocytes, as well as a highly vascularized interfollicular network. In contrast, in the plasma cell type the germinal centers resemble those of follicular hyperplasia, and are composed of a mixture of cleaved and non-cleaved small and large lymphocytes with varying numbers of tingible body macrophages and mitotic figures. The mantle zone is usually intact, and is surrounded by sheets of mature-appearing plasma cells. Transition cases can occur. Both hyaline vascular changes and classic reactive follicles can occur in continuity, both follicle types exhibit the diagnostic onion-skinning of the mantle zones. There is a blurring of the histotypes and these may represent in many cases temporal manifestations of the same disease. Early studies reported the plasma cells to be polytypic in the majority of cases, but in close to 40% of the cases they have been shown to be monotypic, almost always restricted to expression of lambda light chains (Hall *et al.*, 1989).

Since the identification of KSHV in MCD, the understanding of the histology of this disease has changed. While KSHV has been reported in MCD with both hyaline vascular and plasma cell morphology (Larroche *et al.*, 2002), it appears that the majority of cases described in the literature more closely resemble the plasma cell type of MCD. One report indicates that the KSHV-positive cases showed the highest intensity of angiosclerosis and germinal center and perifollicular vascular proliferation, while plasmacytosis is less pronounced than in the KSHV-negative

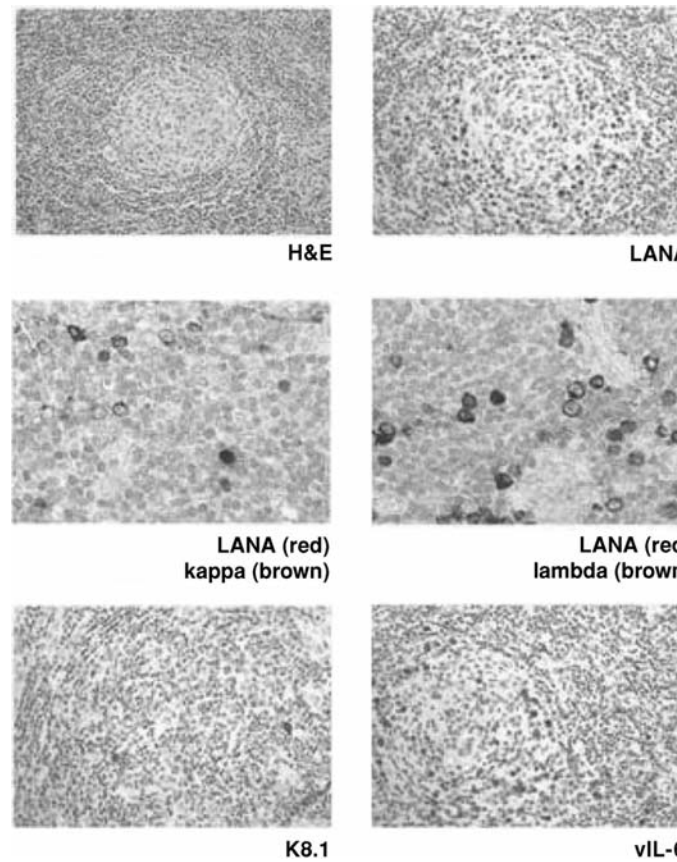

Fig. 50.5. Multicentric Castleman's disease: hematoxylin- and eosin- (H&E) stained section of a lymph node with HIV-associated Castleman's disease showing a single follicle with a large, concentrically arranged mantle zone surrounding a germinal center. The interfollicular area contains a network of small vessels (original magnification 100 ×). Immunohistochemical staining with the following antibodies is shown: LANA (ORF 73), a monoclonal rat antibody was used, showing brown nuclear positivity in cells in the mantle zone (original magnification 100 ×); LANA and kappa, double staining showed nuclear positivity (red staining) in cells that are negative for cytoplasmic kappa (brown staining); LANA and lambda, double staining shows cells that are positive for nuclear LANA (red staining) as well as cytoplasmic lambda (brown staining) (original magnification 400 ×); K8.1, a monoclonal antibody shows cytoplasmic positivity (brown staining) in two cells that are undergoing lytic viral replication (original magnification 100 ×); vIL-6, cytoplasmic staining using a rabbit polyclonal antiserum shows positivity in numerous cells in the mantle zone (original magnification 100 ×).

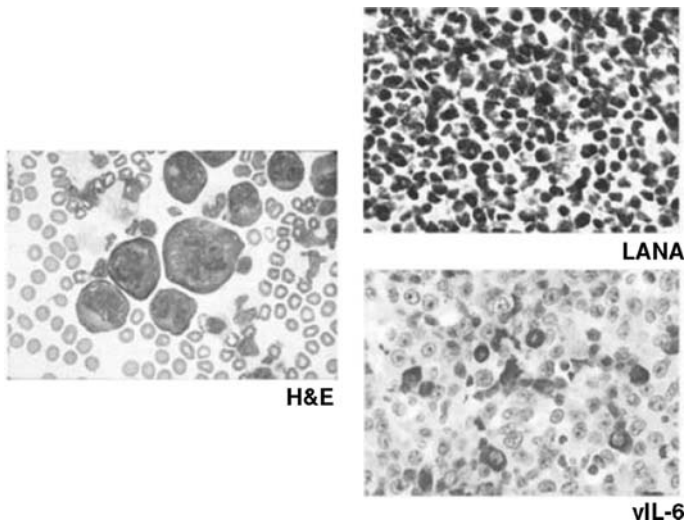

Fig. 50.6. Primary effusion lymphoma: Wright-Giemsa stain air-dried cytocentrifuge preparation of a KSHV-positive primary effusion lymphoma. The tumor cells in this image are considerably larger than normal benign lymphocytes and monocytes. The cells display significant polymorphism and possess moderately abundant basophilic cytoplasm. A prominent, clear perinuclear Golgi zone can be appreciated in several cells. The nuclei vary from large and round to highly irregular, multilobated, and pleomorphic and often contain one or more prominent nucleoli (original magnification 1000 ×). Immunohistochemical staining with the following antibodies is shown: LANA (ORF 73), a monoclonal rat antibody was used, showing brown speckled nuclear positivity in all the neoplastic cells; vIL-6, immunohistochemical staining with a polyclonal rabbit antiserum shows abundant cytoplasmic expression (brown) in many lymphoma cells (original magnification 400 ×).

cases of the plasma cell type (Suda *et al.*, 2001). It now appears that the KSHV-positive cases represent a distinct morphologic variant, resembling more the plasma cell type, but in addition showing the presence of larger cells in the mantle zones, which are approximately twice the size of mantle zone lymphocytes, and characterized by a moderate amount of amphophilic cytoplasm and a large vesicular nucleus containing one or sometimes two prominent nucleoli. These cells have been called plasmablasts, although they frequently have immunoblastic features (Dupin *et al.*, 2000). Expression of IgM can be used to distinguish these plasmablasts from the interfollicular plasma cells that don't express IgM. These cells can be numerous and coalesce.

Immunohistochemistry for KSHV has become a useful diagnostic method for MCD. Monoclonal antibodies to LANA (Dupin *et al.*, 1999) show the presence of KSHV-positive cells, which are the plasmablasts located mostly in the mantle zones of lymph nodes of patients with MCD. These KSHV-infected plasmablasts are B cells that for some unknown reason are monotypic but polyclonal, almost invariably expressing IgMλ (Du *et al.*, 2001). KSHV-positive lymphocytes can also be seen spilling into the germinal centers and in interfollicular areas. One study showed that KSHV-positive endothelial cells can also be found in MCD lymph nodes, in both HIV-positive and -negative patients (Brousset *et al.*, 2001). In addition antibodies to vIL-6 are useful, as this viral protein is also frequently expressed in MCD in scattered plasmablasts surrounding the lymphoid follicles (Cannon *et al.*, 1999a,b; Parravinci *et al.*, 1997; Staskus *et al.*, 1999), and expression of this viral cytokine may confer a worse prognosis (Menke *et al.*, 2002). Lytic antigens are also expressed more frequently in KSHV-infected cells in MCD that in other disorders associated with this virus, suggesting that lytic viral replication may be a feature of MCD (Katano *et al.*, 2000).

Therapy for Castleman's disease remains ill-defined. However, evidence of lytic viral expression has led some to advocate ganciclovir or other antiviral therapy. Indeed, there are small anecdotal reports that indicate responses to ganciclovir (Casper *et al.*, 2004). Other therapies targeting the tissue compartment harboring the virus have also been associated with responses. These include the use of an anti-B-cell antibody rituximab or splenectomy (Corbellino *et al.*, 2001; Lerza *et al.*, 1999; Marcelin *et al.*, 2003).

Primary effusion lymphoma and related lymphomas

Primary effusion lymphomas arise mainly in patients with HIV infection and preferentially involve body cavities and occasionally extranodal sites. These tumors always carry KSHV and are commonly coinfected by EBV. The classical presentation is with signs and symptoms of an effusion in the absence of a tumor mass (Nador *et al.*, 1996). The cells in the effusion are large, have abundant cytoplasm, usually amphophilic to basophilic, and nuclei that range from large, round and regular to highly irregular and pleomorphic, with one or more large prominent nucleoli (Fig. 50.6) (Cesarman *et al.*, 1995). Many cells have plasmacytoid or immunoblastic features. Binucleated or multinucleated cells resemble Reed–Sternberg cells can be seen. Mitotic figures are typically numerous. The immunophenotype is characteristic, as PEL cells express of CD45, CD138 and one or more activation-associated antigens in the frequent absence of B cell-associated antigens and immunoglobulin expression. A B cell origin can be demonstrated by the presence of clonal immunoglobulin gene rearrangements, and the immunoglobulin genes show somatic hypermutation indicating a postgerminal cell stage of differentiation.

Immununohistochemistry shows the presence of LANA and other KSHV proteins in a variable proportion of neoplastic cells, in particular vIL-6. In addition, the majority of PELs are coinfected with EBV, which can be detected by in situ hybridization to EBERs.

Lymphomas containing KSHV can also present as solid tissue masses, usually extranodally, similar to other AIDS-related non-Hodgkin's lymphomas. While some of these lymphomas subsequently develop an effusion, others apparently do not. They usually present as solid extranodal lymphomas and are diagnosed as diffuse large cell, immunoblastic, or anaplastic large cell lymphomas, in which the presence of KSHV in practically all the lymphoma cells could be demonstrated by immunohistochemistry or molecular techniques (Chadburn *et al.*, 2004; Engels *et al.*, 2003). Most of these are immunoblastic in appearance, have a high mitotic rate and variable amounts off apoptotic debris. These lymphomas appear to fall in the spectrum of PEL, as they usually lack expression of B cell antigens and immunoglobulin, they have a similar morphology, and they are frequently co-infected with EBV.

Survival with conventional chemotherapy is dismal, with the reported median survival time less than 6 months (Boulanger, 2005).

Several diseases have been purportedly linked to KSHV. In multiple myeloma patients, the virus was not reported to be in tumor cells but in the associated bone marrow stroma (Rettig *et al.*, 1997; Said *et al.*, 1997). There was the suggestion that production of viral interleukin-6 or cellular interleukin-6 by the stromal cells drove B-cells in the marrow to malignancy. However, many investigators have failed to confirm these findings (Ablashi *et al.*, 2000; Bellos *et al.*, 1999; Brander *et al.*, 2002; Corbellino *et al.*, 1999; Dominici *et al.*, 2000; Drabick *et al.*, 2002; Tarte *et al.*, 1999; Pan *et al.*, 2001). Similarly associations of KSHV with sarcoidosis (Di Alberti *et al.*, 1997) and pulmonary hypertension (Cool *et al.*, 2003) have been reported. Confirmation of a general association with either disease process is lacking (Henke-Gendo *et al.*, 2004; Lebbe *et al.*, 1999; Maeda *et al.*, 2000; Moore, 1998; Regamey *et al.*, 1998; Sugaya *et al.*, 1999).

Conclusions

The human gammaherpesvirus-associated diseases are quite distinct from those associated with other herpesviruses. Rather than tissue destruction in association with lytic infection such as is characteristic of the alpha and beta herpesviruses, the gammaherpesviruses are associated with proliferative diseases and malignancies. The seroprevalence of EBV and KSHV is much more widespread than the associated proliferative diseases. Several of these are most common in immunocompromised patients, but other gammaherpesvirus-associated tumors arise in hosts that are not globally immunocompromised. The host genetic and environmental determinants of disease beyond immunocompromise are poorly understood. Detection of viral nucleic acid or proteins is important in the diagnosis of some of the lymphomas and lymphoproliferative disease. Treatment with virus-specific therapies is still in its infancy. Antivirals that target the viral DNA polymerase such as acyclovir and ganciclovir have very limited if any impact on benign or malignant disease, although there is a hint that these agents may reduce the incidence of tumorigenesis in high risk populations. Two exceptions might be noted: oral hairy leukoplakia clearly responds to antiviral therapy and anecdotal reports suggest that Castleman's disease may also. Adoptive cellular immunotherapies appear to hold promise and have achieved some impressive successes in the bone marrow transplant population in the prevention and treatment of lymphoproliferative disease.

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EBV: immunobiology and host response

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Introduction

The biology and immunology of Epstein–Barr virus (EBV) has continued to fascinate researchers because the lessons learnt provide a platform for understanding the interplay between the biology of this ubiquitous infection, the immune system seeking to restrict its spread and the emergence of a variety of malignancies. As with other gamma herpes viruses, EBV encodes a large set of lytic cycle genes together with a number of latent genes which are associated with expansion of the latent EBV pool in B-lymphocytes. Current evidence suggests that the virus gains entry into the body by infection of B-lymphocytes in the oral cavity via an interaction between the major viral glycoprotein gp340 and the complement receptor CR2 which is expressed on B-cells, although a role for CR2-expressing or non-expressing epithelial and/or T-cells cannot be totally discounted. In either case, evidence suggests that the earliest detectable event following primary infection is the expression of lytic cycle proteins resulting in the release of infectious virus into the oral cavity followed by a generalized seeding of latently infected B-lymphocytes throughout the body. This primary infection results in symptoms of acute infectious mononucleosis (IM) in about 50% of adolescents and is coincident with a marked lymphocytosis (dominated by EBV-specific cytotoxic T-cells) and the appearance of an IgM response to a variety of EBV proteins, most notably the viral capsid antigen, VCA. Current evidence suggests that this cytotoxic T-cell (CTL) response, which includes both CD4+ and CD8+ cells restricts expansion of these latently infected B-cells and results in a long-term carrier state in which there is an equilibrium between the level of secretion of the virus and the number of latently infected B-cells.

Latently infected B cells are of central importance in the overall biology of the virus, and the function of individual latent proteins has been studied in EBV transformed lymphoblastoid cell lines (LCLs). The virus expresses eight EBV latent genes including six nuclear proteins (EBNA1, 2, 3A, 3B, LP and 3C) and two integral membrane proteins (LMP1 and LMP2). Furthermore, virus-infected cells invariably express two small polyadenylated RNAs (EBER-1 and -2) which are often used as a sensitive marker for the presence of EBV within a cell. The nature of the long-term latent infection in vivo has been subject to considerable speculation but is likely to include expression of a limited number of proteins (EBNA1 and LMP1) in a B-cell pool that maintains a phenotype that is poorly recognized by CTL. Thus the virus appears to have evolved so that apart from the clinical effects of IM (which appears to be historically a relatively recent syndrome), most individuals suffer no consequences from carrying a small nucleus of latently infected B-cells which appear to be resistant to CTL recognition and which can, under certain circumstances be reactivated to release relatively low levels of virus into the oral cavity.

The dynamics of the establishment of this small pool of long-lived latently infected B-cells and the CTL response is poorly understood but involves a selection process imposed by the CTL response which recognizes and eliminates B cells with a full complement of latent antigens and a relatively rare differentiation step that permits the emergence of a long-lived memory B cell pool that is resistant to specific lysis and whose phenotype supports the expression of EBNA1 and LMP1. It appears however, that this seemingly elegant balance that ensures the long-term survival of the virus is subject to error on certain occasions resulting in the emergence of EBV-driven B cell malignancies.

These B cell malignancies are classified in terms of the degree of latent antigen expression. Burkitt's lymphoma (BL) is at one end of the spectrum and is characterized by the expression of a single EBV protein (EBNA1 whose phenotype suggests a germinal centre origin (latency 1).

BL cells are well adapted to escape CTL recognition since they have down-regulated expression of class I MHC and of the transporters associated with antigen processing (TAP-1 and/or TAP-2) genes. Furthermore, EBNA1 has a series of glycine-alanine repeat (GAr) sequences that are speculated to exert an inhibitory effect on the endogenous processing of this antigen through class I although it appears this inhibition can be over-ridden in vivo, since both CD4+ and CD8+ EBNA1-specific CTLs have been detected in healthy virus carriers.

Hodgkin's disease (HD) is a second EBV-associated B cell malignancy. In this case, the tumor cell is derived from a post-germinal center B cell and is characterized by expression of EBNA1 and LMP1 and 2 (latency II). It is interesting that the degree of association of HD with EBV is variable according to the histological sub-type ranging from 80% in the case of mixed cellularity to 20% in the case of nodular sclerosing. This difference between histological types presumably reflects the efficiency with which this form of EBV latency has adapted to each of the respective environments associated with these different histological sub-types.

Latency III malignancies have arisen as a result of intense immunosuppression which have prevented the usual efficient culling of B-cells expressing a full spectrum of latent EBV proteins rather than an exploitation of the virus to a new phenotypic niche. These malignancies arise in transplant patients (particularly EBV seronegative graft recipients) and in late stage AIDS patients and in each case the latently-infected B-cell is clearly post-germinal center in origin.

EBV has also adapted to establishing malignancies in a non-B cell environment. Examples of non-B-cell malignancies includes a range of epithelial tumors the clinically most important of which is nasopharyngeal carcinoma (NPC). This malignancy which has a latency II phenotype is most common in south-east Asia, north Africa and Greenland with a low incidence throughout the rest of the world.

The review discusses the role of the EBV-specific CTL response in controlling EBV-infected cells in each of these latency types. Particular attention will be given to newly emerging concepts rather than re-emphasizing historical aspects of EBV immunology which have been well summarized in a series of reviews in the past 10 years (Rickinson and Kieff, 1996; Khanna and Burrows, 2000)

Response during acute infection

The identification of CTL epitopes within EBV proteins was based largely on techniques that favored the definition of $CD8^+$ CTL within EBV latent proteins. In part this has been due to an analysis of the CTL response emerging from *in vitro* cultures of PBMC from healthy immune individuals stimulated with autologous LCLs which express EBV latent rather than lytic proteins. In spite of these limitations and the obvious skewing of defined epitopes towards those in latent proteins, a number of lytic cycle epitopes have been defined and have provided a platform for an analysis of the CTL response seen during acute infection and redistribution of this response in healthy individuals although there are certainly indications that the response during acute compared to silent seroconversion may be fundamentally different. The lymphocytosis seen during acute infection can result in a ten fold increase in T-cell numbers and is characterized by cells with an activated phenotype (CD38, HLA DR and CD69) (Callan *et al.*, 1998; Bharadwaj *et al.*, 2001) (Fig. 51.1). These cells also express surface markers with a memory or effector phenotype (i.e., perforin, CD27, CD45RO), lack of expression of CCR7 (Sallusto *et al.*, 1999; Callan *et al.*, 2000; Hislop *et al.*, 2002) and are known to be susceptible to apopotosis after a brief period of in vitro culture (Moss *et al.*, 1985; Bishop *et al.*, 1985). It is not surprising that, during acute infection, which is characterized by high levels of EBV secretion in the oral cavity and high levels of lytic antigen expression in certain lymphoid tissue, the CTL response is directed largely towards lytic proteins. Immediate early gene products in particular have been shown to generate potent responses when visualized by HLA class I tetramer staining of PBMCs from IM patients (Callan *et al.*, 1998; Hislop *et al.*, 2002). Responses to epitopes from the early gene products, such as those derived from BMLF1 and BMRF1 are generally smaller than responses to the immediate early proteins but can still represent up to 12%

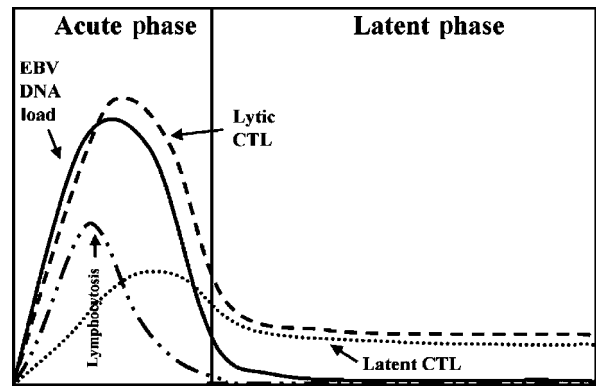
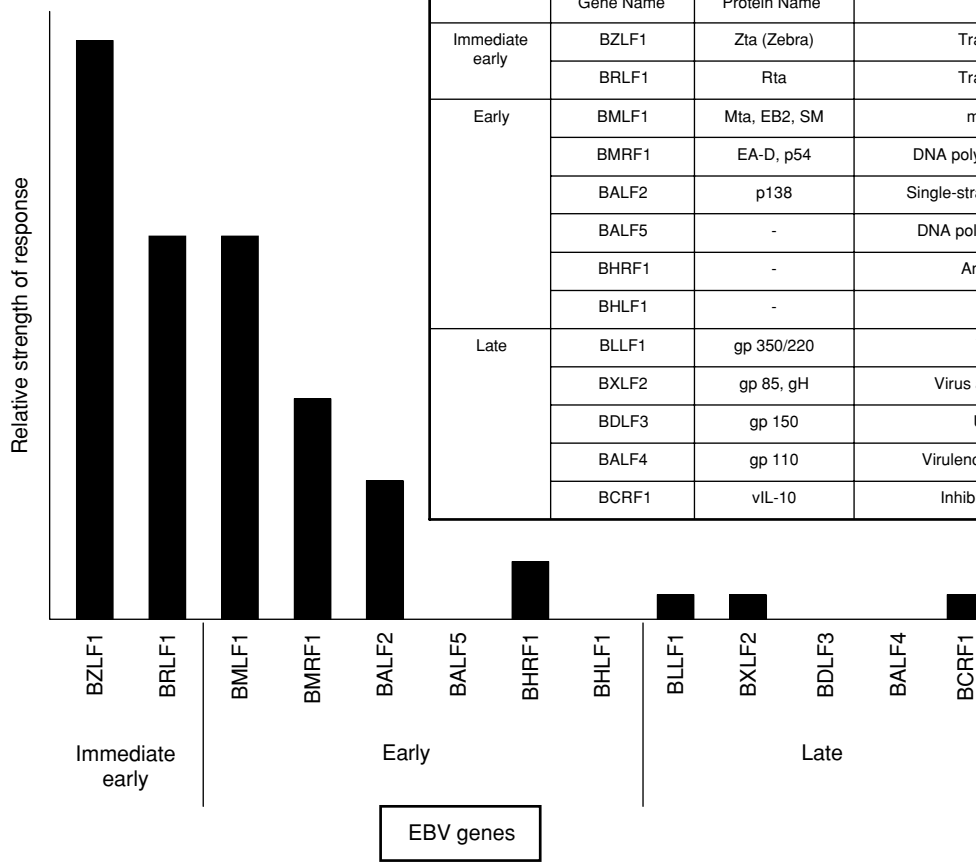


Fig. 51.1. Diagrammatic representation of the dynamics of the activation of a CTL response to EBV lytic and latent proteins in acute primary infection compared to long-term healthy immune individuals. These immune parameters are also shown in relation to EBV viral load and lymphocytosis.



	Gene Name	Protein Name	Function
Immediate early	BZLF1	Zta (Zebra)	Transcription activator
	BRLF1	Rta	Transcription activator
Early	BMLF1	Mta, EB2, SM	mRNA export factor
	BMRF1	EA-D, p54	DNA polymerase processing factor
	BALF2	p138	Single-stranded DNA binding protein
	BALF5	-	DNA polymerase catalytic subunit
	BHRF1	-	Anti-apoptotic protein
	BHLF1	-	oriLyt promoter
Late	BLLF1	gp 350/220	Viral glycoprotein
	BXLF2	gp 85, gH	Virus attachment/penetration
	BDLF3	gp 150	Unknown function
	BALF4	gp 110	Virulence factor/virus maturation
	BCRF1	vIL-10	Inhibits immuneparameters

Fig. 51.2. Diagrammatic representation of the relative strength of EBV immediate early, early and late lytic proteins. The ascribed function of each of these proteins is also listed as an insert.

of the $CD8^+$ population (Annels *et al.*, 2000; Callan *et al.*, 1998; Hislop *et al.*, 2002). Current indications are that the response to late cycle lytic proteins is smaller than the response to either the early lytic cycle proteins or to the latent proteins, although it can contribute up to 3% of the $CD8^+$ response during acute infection (Steven *et al.*, 1996; Annels *et al.*, 2000; Hislop *et al.*, 2001). Overall the relative strength of the CTL response between different EBV lytic proteins (shown diagrammatically in Fig. 51.2) raises the possibility that the magnitude of the response is dictated by their order of synthesis. Although there is the general impression that the overall response in acute infection is extremely focused, it needs to be stressed that there are a relatively modest number of lytic cycle epitopes defined and the contribution of CTL reactivity during acute infection within the majority of these proteins is undefined. However, it should be pointed out that recent results have cast some doubt on the in vivo significance of functional significance of the T cell responses to lytic proteins. These reservations are based on the observation that expression of lytic proteins may result in the down-regulation of both class I MHC and TAP proteins (Ressing *et al.*, 2005).

As mentioned previously, much of what is known about the immune parameters established during primary infection has been inferred from individuals undergoing acute IM. A study (Silins *et al.*, 2001) of a small cohort of adolescent EBV-seronegative individuals undergoing asymptomatic primary infection has provided some insight into the immunobiology of the silent primary infection experienced by the majority of individuals. Such individuals did not develop a significant T-cell lymphocytosis in spite of the presence of EBV viral loads comparable to that seen during acute infection raising the possibility that symptomatic seroconversion is associated with the release of cytokines from the activated T-cell expansions rather than developing as a result of virus-induced pathology. These

studies reveal a striking difference in the degree of TCR diversity seen in those undergoing symptomatic EBV seroconversion compared to those undergoing silent seroconversion. It appears that an oligoclonal TCR repertoire during primary infection is associated with the appearance of clinical symptoms whereas a polyclonal response predisposes towards silent seroconversion..

Response in healthy virus carriers

As the T-cell lymphocytosis resolves during convalescence, there is a reduction in the number of T-cells with activation markers and the relative contribution of the CTL response directed towards latent and lytic proteins is diminished (Fig. 51.1). Although a wide range of HLA alleles have been shown to present EBV T cell epitopes, in most cases, epitope selection is highly allele specific. There are, however, a number of epitopes that show degeneracy in HLA restriction and are immunogenic in HLA mismatched individuals, thus broadening their potential population coverage if included in a CTL-based EBV vaccine. This includes the PYLFWLAAI epitope from LMP2A that is moderately to strongly immunogenic in HLA A*2301$^+$ individuals and weakly immunogenic in most individuals expressing the more common HLA A*2402 (Burrows *et al.*, 2003). In an earlier example, an EBNA 6 epitope was shown to be presented on three closely related subtypes of the B27 allele (Brooks *et al.*, 1993).

Furthermore, it is clear that the response in healthy immune individuals is not a miniturized version of that seen during acute infection but has been specifically selected to control cell-virus relationships established during long-term latent infection during which the level of EBV secretion is relatively modest and the proportion of latently-infected B cells is less than 1% of that present during acute infection. Analysis of CTL responses using tetramer staining, ELISPOT assays and cytokine production has allowed an estimate of the relative immunodominance in responses in healthy EBV immune individuals. This analysis has revealed that the intensity of the lytic antigen-specific response has waned compared to that seen during acute infection and that the response to latent antigens has increased. It is curious that this increased response is not consistent across all EBV latent proteins but is focused particularly to epitopes within EBNA3A, 3B and 3C with a subdominant response in the case of the two membrane-spanning proteins, LMP1 and 2 and a level of response towards EBNA1 that is controversial (see below). Overall, this relative level of immunodominance within the EBV latent proteins does not appear to be determined by their order of synthesis suggesting that other immunobiological factors are more important. The nuclear location of the EBNA3 proteins ensures their efficient processing and presentation through the endogenous pathway. However, in the case of LMP1 and 2 proteins, evidence suggests that their structure and location frequently favors processing through TAP-independent pathway(s). For example, LMP2 is a membrane-spanning protein with a 119 amino acid cytoplasmic N-terminus domain followed by 12 membrane-spanning segments with minimal, if any projection into the cytoplasm, and a 27 amino acid cytoplasmic C-terminus domain. Several reports (Khanna *et al.*, 1996; Lee *et al.*, 1996) have indicated that many, but not all of the epitopes within LMP2 are TAP-independent and that these epitopes lie within the transmembrane stretches of the protein whereas the TAP-dependent epitopes are markedly less hydrophobic (Lautscham *et al.*, 2003). These observations raise the possibility that processing and presentation by a TAP-independent, proteosome-dependent pathway might be linked either to the transmembrane location of this protein or its extreme hydrophobicity.

Role of CD4+ and CD8+ CTL in control of EBV infection

Under the experimental conditions used for the identification of EBV CTL epitopes, class I-restricted CD8$^+$ CTL effectors have been more commonly recognized than CD4+ effector cells. During IM, expansions of activated CD4$^+$ T-cells are observed; however relatively little is known about their specificity or importance in controlling EBV replication either in terms of provision of direct effector function or as a source of immunological help. Direct *ex vivo* cytolytic assays from IM patients have demonstrated CD4 T-cell-mediated EBV-specific cytotoxicity, and in one case this response has been mapped to an epitope in BHRF1 (Schmidt and Misko, 1995). Given the importance of CD4$^+$ T-cells in maintaining CD8$^+$ T-cell responses, it will be of great value to further characterize these cells and their target epitopes to define their role in IM.

In healthy immune individuals, CD4+ responses to epitopes from BHRF1, EBNA1 and EBNA3C have been described and cells specific for epitopes derived from these proteins have been found to be cytotoxic effectors and can produce interferon-γ (Schmidt and Misko, 1995; Leen *et al.*, 2001) regardless of the expression of the co-stimulatory molecules CD27 or CD28. Overall, the hierarchy of CD4+ CTL responses appears to be EBNA3C > EBNA1 > LMP2 >> LMP1 while in contrast the hierarchial

T-helper responses for the same proteins is EBNA1, EBNA3C >> LMP1, LMP2 (Leen *et al.*, 2001). It should be pointed out that the CD4+ assays to detect helper function are based on the release of interferon-gamma and are detecting T-helper activity of a kind thought to reflect CD8 T-cell induction rather than T-cell help associated with a humoral response which would involve analysis for cytokines such as IL-4, IL-5 and IL-13.

The CD4+ and CD8+ CTL response to EBNA1 has received particular attention in recent years. As already mentioned, the pronounced GAr repeat sequences have been reported to inhibit processing and degradation of this protein. Interestingly, most of these CD8+ responses are directed towards epitopes that are COOH-terminal of the GAr domain. The CD8+ response is not derived from full length EBNA1 but from proteins that are prematurely truncated during translation or mal-folded after translation (referred to as defective ribosomal products or DRiPs (Tellam *et al.*, 2004). These DRiPs are apparently subject to proteosomal- and serine protease- mediated (Voo *et al.*, 2004) degradation and class I presentation. In contrast, it is likely that the CD4 response is derived from full length EBNA1 released from dying cells which is subsequently exogenously processed through the class II pathway.

Role of CTL effector cells in resolution of acute IM

This background information on the CTL responses during primary EBV infection and in healthy immune individuals provides some insight into the protective role of these individual responses in resolution of disease. This issue is of central importance in designing a prophylactic vaccine to prevent the clinical symptoms of IM or a therapeutic vaccine to induce regression of the EBV-associated malignancies. The dynamics of latent compared with a lytic antigen-specific responses in acute EBV infection suggests that the latter strong response is incapable of reducing clinical symptoms and might, be driving symptomology. Generally, waning clinical symptoms are coincident with an increasing latent CTL response (Fig. 51.1). Furthermore, there are several studies that suggest that the induction of a potent CTL response to latent proteins might be the preferred strategy in relation to an IM vaccine and that a response to lytic proteins might be associated with pathology. Firstly, in a detailed study of a patient treated by adoptive immunotherapy of autologous CTL, it was noted that there was a correlation between the induction of a strong latent antigen-specific response and the cessation of disease, while a sustained lytic response was coincident with disease progression (Sherritt *et al.*, 2003). Secondly, a study of HLA identical individuals, one of whom sustained prolonged clinical symptoms from primary EBV infection and the other who recovered after a brief period, has been useful in ascribing the link between the CTL response and the severity of acute symptomology. It was clear that rapid recovery was associated with the induction of a broad latent antigen-specific response and that acute disease corresponded with a sustained and focused lytic-antigen response. Thus, provided that we can assume that the lessons from EBV infection of B lymphocytes in vitro are relevant in vivo and that the initial event in primary infection is contact between the virus and a B lymphocyte, a strong case can be mounted in favor of directing an IM vaccine towards latent rather than lytic proteins. Such a vaccine would restrict the latently-infected B cell pool expansions some of which presumably progress towards expression of lytic proteins which appear to be responsible for the lymphocytosis associated with acute infection.

T-cell receptor usage

It is now clear that acute EBV infection is associated with dramatic perturbations within the peripheral TCR repertoire, particularly within the $CD8^+$ compartment (Callan *et al.*, 1996). Prospective studies on IM patients that have investigated the clonal composition and dynamic regulation of the EBV-specific CTL response have provided an important opportunity to track the developmental process that T-cells undergo from primary to persistent infection (Silins *et al.*, 1996; Callan *et al.*, 1998; Silins *et al.*, 1997). Importantly, these studies have demonstrated different levels of TCR diversity selection depending on the viral epitope that is the target of the response. For example, the TCR repertoire utilized in the response to two HLA-B8-restricted epitopes from the latent antigen EBNA3A (FLRGRAYGL and QAKWRLQTL) was found to be oligoclonal in one IM patient, with preservation of distinct clonotypes into the memory T cell pool (Silins *et al.*, 1996). In contrast, T-cell populations raised against an HLA-B8-binding epitope from the lytic antigen BZLF1 (RAKFKQLL) was highly diverse in several IM patients, with no dramatic signs of repertoire focussing over time (Silins *et al.*, 1997). Studies by Callan *et al.* have indicated that the larger the $CD8^+$ clonal burst size during IM, the greater the decay observed after symptoms resolve (Callan *et al.*, 2000; Callan, 2003). Thus clonal dominance and immunodominance are less marked in healthy virus carriers than in IM patients. These findings may be related to a limit in the number of cell divisions a T-cell clone can undergo; thus the progeny of clones that have expanded massively during a primary immune

response may be more prone to die as a result of senescence (Callan, 2003). Overall, the developing picture in IM is that a broad range of TCRs are selected and maintained within the composite CTL response against natural EBV infection. A relatively diverse T-cell response may be especially important in the quick recovery from IM and the establishment and maintenance of effective lifelong CTL control.

In the persistent virus carrier state, the influence of EBV infection on the peripheral TCR repertoire remains significant but is much less dramatic than during the acute infection. EBV-specific CTL frequencies are surprisingly high in healthy EBV-seropositive individuals, presumably as a consequence of antigen persistence. Since the CTL response to some EBV epitopes is oligoclonal, it was proposed that infection with viruses such as EBV and CMV contribute to the decreasing diversity in the $CD8^+$ T-cell repertoire that occurs with age. Indeed, a report from Silins *et al.* (1998) supports this contention by demonstrating that the frequently monoclonal response to the EBNA3A epitope FLRGRAYGL is often large enough to dramatically skew the entire TCRBV6 blood repertoire towards oligoclonality.

As in acute IM, EBV-specific $CD8^+$ T cell responses can be either highly focused or restricted in TCR usage depending on the target viral epitope, and it is not clear what controls this variability. For example, a high degree of clonotypic diversity has been shown for the potent CTL response directed towards the HLA B8- and B*4002-binding epitopes from the BZLF1 lytic antigen, with multiple TCRs sharing very few obvious structural features employed in each case (Couedel *et al.*, 1999; Silins *et al.*, 1997). In contrast, a recent analysis of the equally strong response to an HLA B*3501-binding BZLF1 epitope has revealed that a single CTL clonotype often dominates this response in $B3501^+$ individuals (Miles *et al.*, 2005), with almost identical TCRs used by some unrelated individuals (unpublished observation). The basis for the selection of such immunodominant or "public" TCRs is unclear, although it does not appear to be the result of preferential expansion of high avidity TCRs. Other selection pressures may be involved such as cross-reactive stimulation with another foreign epitope or with a positively selecting self-peptide. Another recent proposal based on the crystal structure of an EBV-specific "public" TCR suggests that immunodominant T cell antigen receptors are selected as a result of certain structural properties that may confer better signaling upon ligation (Kjer-Nielsen *et al.*, 2003).

Negative selection pressures can also theoretically limit the diversity of T-cell responses, such as might occur if a viral epitope was highly homologous with a self peptide. However, this is clearly not the explanation for the dominance of the highly conserved TCR structure that is commonly utilized in response to the B8-binding FLRGRAYGL epitope. This TCR can mediate cross-reactive lysis of EBV-positive and EBV-negative target cells expressing the HLA B*4402 alloantigen (Burrows *et al.*, 1994). Not surprisingly, T-cells with this particular TCR are not detected within the EBV-induced memory CTL population in individuals who co-express HLA B8 and B*4402 due to their potential for self-reactivity (Burrows *et al.*, 1995). Interestingly, however, such individuals do still make a response to the FLRGRAYGL–B8 complex through a variety of different TCRs, illustrating the flexibility and reserve strength of the TCR repertoire in the response to a target epitope.

Virus-driven immune modulation

EBV, like many other herpes viruses, has successfully adapted as a persistent infection by developing various strategies to avoid the potentially hostile effects of host immunity. One such adaptive strategy employed by many oncogenic viruses (such as EBV) involves restricted expression of viral genes, thereby minimizing the potential recognition of target antigens (as seen in many EBV-associated malignancies). On the other hand, viruses (including EBV) are also known to interfere more directly with the host immune response by encoding viral genes, that are homologous to cellular effector molecules. Candidate immune evasion strategies against CTL-mediated control have been recognized which act at the level of cytokine regulation and antigen processing and presentation (Cohen, 1999; Khanna *et al.*, 1995; Spriggs *et al.*, 1996). In the last few years, a number of EBV encoded immunomodulators have been identified within the lytic and latent phases of viral infection which may interfere with virus-specific T-cell responses and enhance EBV-infected B-cell proliferation, thus facilitating pathogen dissemination and survival early in the infectious process.

Modulation of the cytokine network

Previous studies have shown that cytokines contribute directly towards the clearance of viral infections. To counter this potential threat, many viruses have evolved to express novel homologues of these cytokines that can act as antagonists. These homologues can be either positive or negative regulators of T cell-mediated immune responses. The EBV encoded, BCRF1 protein expressed during lytic infection exhibits 78% identity to the deduced amino acid sequence of human IL-10 and shares a similar function to its human homologue (Moore *et al.*, 1990). Recombinant BCRF1, like human IL-10 negatively regulates IL-12 which

promotes IFN-γ production by T-cells. In addition, BCRF1 also abrogates the inhibitory capacity of T-cells which blocks the outgrowth of EBV-infected B-cells. This effect may be mediated through suppression of T-cell activation-induced IL-2 and IFN-γ production (Bejarano and Massuci, 1998). Apart from its effect on IL-2 and IFN-γ production, it has also been proposed that BCRF1 acts directly on T cells to inhibit co-stimulatory signals mediated via B7 receptors such as CD28 or CTLA-4 (Muller *et al.*, 1998). Since BCRF1 is mainly expressed during the replicative phase of the virus cycle, the role of IL-10 in the modulation of latent infection has remained largely unresolved until recently. Studies carried out by Marshall and colleagues showed that LMP1 preferentially activates regulatory T-cells that secrete IL-10 (Marshall *et al.*, 2003). These IL-10 responses inhibit T cell proliferation and IFN-γ secretion by EBV-specific CD8+ T cells.

Another example of modulation of the cytokine network by EBV comes from studies by Strockbine and colleagues (Strockbine *et al.*, 1998) who have identified a novel EBV-encoded modulator of colony stimulating factor (CSF1). Their studies showed that BARF1 protein, when added into the culture medium, neutralizes the proliferative effects of human CSF-1 which is a pleiotropic cytokine best known for its differentiating effects on macrophages. Thus BARF1 may function to modulate the host immune response to EBV infection by blocking CSF-1 function through inhibition of interferon a (IFN-a) secretion from mononuclear cells (Cohen and Lekstrom, 1999) which is the first cytokine produced in response to virus infection. Recent studies have shown that IFN-a activates the p53 gene in virus-infected cells leading to apoptotic death of infected cells (Takaoka *et al.*, 2003). Based on these studies, we hypothesize that BARF1-mediated blockade of IFN-a would protect EBV-infected cells from death during the early stages of infection thus promoting latent infection. In addition to its potential role in promoting latent infection, the role of BARF1 in modulating innate and adaptive immunity should not be ignored since IFN-a activates NK cells and is necessary for both NK cell blastogenesis and cytotoxicity during herpes virus infections (Brion *et al.*, 1995). Furthermore, preliminary studies carried out in our laboratory have shown that recombinant BARF1 protein can inhibit activation and expansion of memory T-cells from healthy virus carriers (S. Pai and R. Khanna, unpublished observations).

Regulation of antigen processing and presentation

T-cell-mediated immune control of EBV infection can act at either latent or lytic phases. The pattern of viral gene expression at each phase determines the potential target antigens. When considering immune evasion strategies for latent infection, EBNA1 remains one of most comprehensively studied proteins. Levitskaya and colleagues have shown that EBNA1 resists CTL mediated immune recognition through a unique inhibitory mechanism which blocks endogenous processing of CTL epitopes within this antigen (Levitskaya *et al.*, 1995, 1997). It was proposed that this GAr sequence within EBNA1 may influence the folding pattern of this protein and affect its capacity to associate with various components of the ubiquitin/proteasome pathway, including ubiquitin conjugation enzymes and/or regulatory subunits of the proteasome. More recent studies have shown that this inhibitory effect may be overridden in certain types of epithelial cells (Jones *et al.*, 2003). When EBNA1 is expressed in immortalized epithelial cells (SSC12F, SVK), it not only inhibits cell growth but is also endogenously processed through the class I pathway for immune recognition by CD8+ T-cells. On the other hand, neither of these phenomena is observed when EBNA1 is expressed in fully transformed epithelial cells (Hela, Ad/AH).

LMP1 is another latent protein known to modulate the function of immune regulatory proteins in B-cells. This protein has recently been recognized as a functional homologue of human CD40 (Uchida *et al.*, 1999). First evidence for a potential role of EBV latent antigens in the regulation of MHC class I expression came from the observation that LMP1 was capable of reversing down-regulated expression of TAP and HLA class I in BL cells which are characterized by defective antigen processing (Khanna *et al.*, 1995). Recent studies from our laboratory have shown that the c-terminal domain of LMP1 plays an important role in translocating relB from the cytoplasm to the nucleus with a consequent effect on the upregulation of antigen processing genes (Pai and Khanna, 2002). This observation raises the intriguing question of why an EBV protein has evolved with the capacity to up-regulate antigen processing function in the host cells, thereby potentially increasing the chances of its elimination by virus-specific CTLs. A possible explanation is that the virus uses the LMP1 protein to hyper-regulate the antigen processing function of B-cells, and that this not only results in efficient presentation of viral peptides, but also increases the levels of self peptides presented by these cells. Increasing self peptide presentation has two potential consequences (i) these self peptides may compete with viral peptides thus reducing the levels of EBV peptides presented to CTLs thereby allowing immune escape or alternatively (ii) increased self peptide presentation may also contribute towards the development of autoimmune reactivity which is often seen as one of the symptoms in individuals

with acute EBV infection. Another plausible hypothesis to explain why EBV modulates antigen processing gene expression via LMP1 involves the long-term host-virus relationship whereby some advantage is gained from deliberately enhancing the chance of EBV-infected B-cells being lyzed by immune surveillance thus protecting the host from an overwhelming infection and maintaining a stable host-virus relationship.

Although much of the emphasis on the immune control of EBV has focused on CD8+ T-cells, recent studies have demonstrated that CD4+ T-cells also play an important role in both primary and latent EBV infection (Precopio *et al.*, 2003). It is not surprising that EBV has evolved immune evasive strategies to counter the potential threat from this effector arm of the immune system. Thus Spriggs and colleagues (Li *et al.*, 1997) have proposed a novel mechanism of blocking peptide presentation through the MHC class II pathway. They showed that, during the late lytic cycle, EBV encodes a type II membrane glycoprotein that specifically binds to the ß chains of MHC class II particularly the HLA DR heterodimer both intracellularly as well as on the cell surface resulting in the retention of MHC class II molecules in the ER thus blocking presentation of class II-restricted T-cell epitopes. Interestingly, subsequent studies have shown that BZLF2 specific antibodies that block the interaction of this protein with MHC class II can also prevent the infection of MHC class II positive B lymphocytes (reviewed in (Spear and Longnecker, 2003). It is possible therefore that BZLF2 plays a dual role, modulating class II-restricted antigen presentation as well facilitating infection of class II positive cells.

T-cell control of EBV-associated malignancies

The EBV-associated malignancies have evolved unique strategies to evade immune recognition allowing them to expand in the face of an existing EBV-specific CTL response (Khanna, 1998; Khanna and Burrows, 2000). Thus, in the case of endemic BL, viral gene expression is restricted to EBNA1 with a phenotype resembling that of resting cells. This combination of properties makes these cells highly resistant to T-cell-mediated immune recognition rendering them incapable of stimulating an EBV or allospecific T-cell response (Rooney *et al.*, 1995). This non-immunogenic phenotype of BL cells is linked to down-regulated expression of MHC class I and TAP-1 and/or TAP-2 genes, whereas expression of the proteasome genes, LMP2 and LMP7 is normal in most cases (Khanna *et al.*, 1994, 1995). It is important to mention here that loss of antigen processing function is not only seen in EBV-positive BL cell lines but also in EBV-negative BL cells. Thus it is likely that the CTL resistant phenotype of BL is a reflection of the nature of the cell type from which the malignant cells are derived. Indeed, Gregory and colleagues have identified normal B-cells which display a phenotype identical to BL cells (Gregory *et al.*, 1987).

While considerable significance has been placed on the apparent loss of MHC class I-molecules in the immune escape of tumor cells, evidence from other human tumors has accumulated that CD4+ T-cells can also play a critical role in immune surveillance (Topalian *et al.*, 1994). Surprisingly, analysis of MHC class II-restricted antigen processing function in TAP-deficient BL cells has revealed that EBV-specific CD4+ CTLs can efficiently recognize these tumor cells (Khanna *et al.*, 1997). Furthermore, in contrast to the consistently low levels of surface MHC class I expression on BL cells, MHC class II expression is normal and quite comparable to that on EBV-transformed normal B-cells. Consistent with these observations, BL cells also show normal levels of the HLA DMB gene product which is an essential component for class II processing. The importance of these studies has been further strengthened by the observation that CD4+ EBNA1-specific CTLs from healthy virus carriers can efficiently recognize not only virus-infected normal B-cells but also BL cells (Munz et al., 2000; Paludan *et al.*, 2002).

In spite of distinct pathologies, NPC and HD share a number of phenotypic features which provide a unique opportunity to study the immune responses to viral antigens expressed in these malignancies. Molecular analysis of fresh biopsies and laboratory-established tumor lines indicate that malignant cells in both NPC and HD express normal levels of HLA class I and TAP1/2 (Khanna *et al.*, 1998; Lee *et al.*, 1998) and express only a limited number of latent proteins (LMP1, LMP2 and EBNA1). A number of studies have shown that, as in the case of healthy virus carriers, the EBV-specific CTL repertoire in HD and NPC patients is strongly focused through the EBNA3A, EBNA3B and EBNA3C proteins and thus have a limited capacity to control these tumors in vivo. Lee and colleagues conducted an exhaustive analysis of EBV-specific T-cell responses in a large panel of HD patients which showed no obvious suppression of the EBV-specific T-cell responses in HD patients when compared to the healthy virus carriers (Lee *et al.*, 1998). These observations were in direct contrast to earlier studies which showed that HD patients often possess a generalized defect in cell-mediated immunity, including impaired responses to mitogens and a decreased capacity of T-cells to respond in a mixed lymphocyte response (Slivnick *et al.*, 1990). Although the precise reason for this loss of T-cell immunity is not known, a recent study by

Marshall and colleagues have shown that the $CD4^+$ T-cell response to the LMP1protein is dominated by regulatory T-cells which express high levels of IL-10. These responses inhibited T-cell proliferation and INF-γ production by both mitogen and EBV antigens (Marshall *et al.*, 2003). Furthermore, Dukers and colleagues have also shown that LMP1 includes two novel retrovirus homologous sequences within the transmembrane domain which showed strong inhibition of T-cell proliferation and NK cell cytotoxicity (Dukers *et al.*, 2000). These authors proposed that HD or NPC tumor cells may actively secrete LMP1 and thus mediate immunosuppressive effects on tumor infiltrating lymphocytes.

Future prospects for an EBV vaccine

Our understanding of the immune response to EBV appears to be at a point where serious consideration can now be given towards a vaccine to at least some of the EBV-associated diseases. It appears that the best prospects relate to allowing the development of vaccine to prevent the clinical symptoms of IM. As already discussed, a strong argument can be drawn that this vaccine should be directed towards activating a response to epitopes within EBNA3A, 3B and 3C. The hope is that the same vaccine might be effective against the emergence of PTLD in transplant patients. The method of delivering this vaccine is open to question, but given the fact that the recipients of an IM vaccine will be largely healthy adolescents, it is unlikely that a live vaccine delivery system will be used.

As already discussed, the latent antigens expressed in the latency II diseases (NPC and HD) are poorly immunogenic. This consideration, when seen in concert with the fact that these patients are suffering a life-threatening disease means that live vaccine delivery vectors might be acceptable.

There appears to be little prospect for the development of an effective vaccine against latency 1 diseases. This conclusion is based on the relative low frequency of these malignancies and their non-immunogenic phenotype.

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52

Immunobiology and host response to KSHV infection

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Introduction

The interplay between malignancy, infection and immunity is best illustrated by the neoplasms related to KSHV (Boshoff and Weiss, 2002): Kaposi sarcoma (KS) is approximately 100 times more common during immunosuppression and can be resolved when iatrogenic immunosuppression is stopped (Euvrard *et al.*, 2003) and during highly active antiretroviral treatment (HAART) of HIV-1 infected individuals (Boshoff and Weiss, 2002). Primary effusion lymphoma (PEL) and plasmablastic multicentric Castleman's disease (MCD) also occur predominantly during immunosuppression. Like other gammaherpesviruses, KSHV persists as a latent episome in B-lymphocytes (Ambroziak *et al.*, 1995; Cesarman *et al.*, 1995; Renne *et al.*, 1996), without provoking host responses that would eliminate infected cells. KSHV acquired a fascinating repertoire of decoys to trick the host immune response enabling establishment of lifelong infection in humans with very few clinical manifestations. When the balance between viral infection and host immunity is disturbed, some of the molecular pathways employed by KSHV to evade host immune responses are directly involved in driving oncogenesis (Moore and Chang, 2003). KSHV is an excellent model to study the coevolution of pathogen attack and mechanisms of host counter attack.

KS is most aggressive in the immunosuppressed and resolves with partial restoration of the immune system (Gill *et al.*, 2002). Since the introduction of HAART, there has also been a dramatic fall in the incidence of KS (Jacobson *et al.*, 1999). Although non-immune mechanisms may contribute to this drop in KS cases and the resolution of established lesions, it is reasonable to propose that immune reconstitution is a major factor in the control of this neoplasm (Box 52.1).

We are only starting to understand how KSHV avoids these host responses, which viral epitopes are targets for adaptive immune responses, and how anti-KSHV immunity is altered during immunosuppression (Table 52.1).

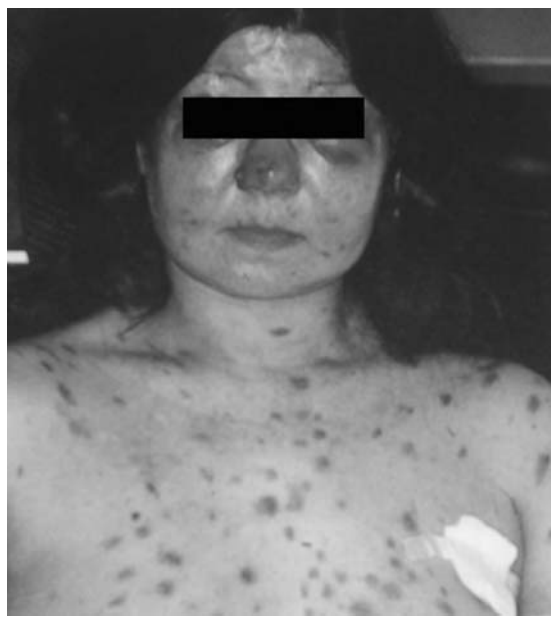
Primary infection

It is thought that KSHV is mainly transmitted by oral exposure to infectious saliva (Pauk *et al.*, 2000), suggesting that mucosal cells are the first port of call. These could be mucosal-associated dendritic cells, macrophages, lymphocytes and/or epithelial cells. KSHV can infect and establish latency in CD34+ hematopoietic progenitors, macrophages and B-lymphocytes in experimental models (Dittmer *et al.*, 1999; Bechtel *et al.*, 2003; Luppi *et al.*, 2005; Wu *et al.*, 2006), but the exact cell type that is predominantly infected at viral exposure is still unknown. KSHV is also present in cells of the endothelial lineage (EC), specifically cells differentiating towards lymphatic endothelium (Dupin *et al.*, 1999; Wang *et al.*, 2004). This microvascular environment is specifically adapted to rapidly eliminate invading pathogens and KSHV must therefore have evolved specific mechanisms to replicate successfully in this niche.

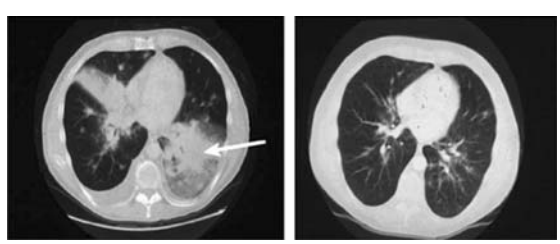
Case reports and epidemiological surveys provide some clues to the consequences of an inadequate immune response to primary KSHV infection: Although primary KSHV infection among immunocompetent individuals can be symptomatic (Andreoni *et al.*, 2002), the development of KSHV-related malignancies is generally associated with immunosuppression. Lymphadenopathy associated with microscopic KS lesions (that is expansion of KSHV-infected EC), occurs in HIV-1 infected individuals, who are thought to be exposed to KSHV for the first time (Oksenhendler *et al.*, 1998). This infers that the lympadenopathic KS seen in African children may also represent primary infection with KSHV, similar to childhood Hodgkin's disease

Box 52.1. Kaposi's sarcoma, immunity and anti-retroviral treatment

For most individuals, living with KSHV is uneventful. However, during immunosuppression the consequences can be severe.



In 1981, Kaposi's sarcoma (KS) became the sentinel of the AIDS epidemic. During the 1980s, prior to the use of any antiretroviral treatment, KS often presented as widespread lesions affecting skin, mucosal surfaces and sometimes internal organs. Periorbital edema was also frequently seen with advanced HIV-1 infection. (Image kindly provided by A. E. Friedman Kien.)



How does HAART prevent KS?

Since the introduction of HAART, the incidence of KS has decreased dramatically, and HAART also leads to rapid KS resolution. The computed tomographic lung scans (Image kindly provided by N. Dupin) show a patient with a large pulmonary KS lesion (left panel) and the improvement in this patient during HAART (right panel).There are several mechanisms by which HAART could lead to KS resolution:

HAART leads to immune reconstitution against KSHV (Wilkinson *et al.*, 2002; Bourboulia *et al.*, 2004). This hypothesis could also explain the resolution of Kaposi's sarcoma in HIV-negative post-organ transplant individuals when immunosuppressive drugs are stopped.Both humoral and cellular immunity might contribute to this.

HIV-1 circulating Tat levels and HIV-1 induced immune activation (including levels of circulating cytokines) are reduced on HAART leading to KS regression (Ensoli *et al.*, 1990; Gallo, 1998). This could explain the often rapid resolution of KS when HAART is initiated, but not the resolution of KS in HIV negative individuals when immunosuppression is stopped (e.g., after an organ transplant).The observation that proangiogenic growth factor (e.g. vascular endothelial growth factor, VEGF) levels decrease during HAART and correlate with KS resolution, supports this theory.

Protease inhibitors (PIs) have a direct anti-spindle cell and/or anti-angiogenic effect (Sgadari *et al.*, 2002) PIs block KS spindle cell growth and angiogenesis in experimental models. Retrospective studies showing that HAART leads to a decreased incidence of Kaposi's sarcoma -and to the resolution of established lesions- were all conducted at a time when PIs were routinely used as part of HAART. However, because of side-effects their use in HAART is now more restricted. Non-PI containing combination anti-retroviral therapy also leads to KS resolution and the incidence of KS remains low in the UK and USA (Gill *et al.*, 2002). However, reports that KS can relapse when a PI-containing regimen is substituted for a non-PI containing regimen (Bani-Sadr *et al.*, 2003) do suggest that PI's directly, or indirectly, play a role in the control of KSHV-infected EC proliferation.

thought to be due to inadequate control of primary EBV infection (Macsween and Crawford, 2003). Furthermore, KS develops more commonly in HIV-1 infected individuals who acquired KSHV after primary HIV-1 infection (Goudsmit *et al.*, 2000), suggesting that an inadequate host response during primary KSHV infection confers the propensity of infected EC to expand, and that EC are one of the first KSHV targets during primary infection. Circulating spindle cells, expressing endothelial and macrophage markers, have been identified from healthy

Table 52.1. Overview of host responses to KSHV infection and future questions

	Cell type	Major findings[a]	Major questions
INNATE IMMUNITY	Dendritic cells	• KSHV infects DC *in vitro* and impairs their antigen presenting properties (Reppocciolo *et al.*, 2006) • Decreased number of pDC in AIDS-KS (Stebbing *et al.*, 2003b) and mDC and pDC in classic KS (Della Bella *et al.*, 2006)	Determine the mechanisms employed by KSHV to regulate DC maturation and function
	Natural Killer cells	• Restoration of NK function correlates with KS resolution on HAART (Sirianni *et al.*, 2002) • KSHV protects KSHV-infected cells from NK cell attack (reviewed in Orange *et al.*, 2002)	Investigate the role of KSHV driven NK cells in control of infection and KS development
	Complement	• Essential for MHV68 control (Kapadia *et al.* 2002) • KSHV ORF4 is a regulator of complement activation (Spiller *et al.* 2003)	Determine the role of complement in KSHV infection
ADAPTIVE IMMUNITY	Cytotoxic T cells	• KSHV sepcific CTL epitopes identified[b] • CTL restoration on HAART correlates with viral clearance (Bourboulia *et al.* 2004)	Determine KSHV specific CTL phenotype and identify further CTL epitopes
	T helper cells	• $CD4^+$ T cells proliferate in response to KSHV (Strickler *et al.* 1999) • $CD4^+$ T cell count below 200/mm^3 is a risk factor for KS development[c]	Determine CD4+ T cell KSHV epitopes and subsets
	B cells	• LANA, K8.1, and ORF65 elicit significant humoral responses[d] • Increase in number of individuals with detectable anti K8.1 responses coincides with plasma KSHV clearance during HAART (Bourboulia *et al.* 2004) • KSHV neutralizing antibodies reduced in AIDS-KS (Kimball *et al.* 2004) • Polymorphisms of FCγRIIIA confer protection against KS (Lehrnbecher *et al.* 2000)	Investigate the neutralizing and effector functions of anti-KSHV antibodies

[a] Key references are given. When more than two references are necessary, they are shown as footnotes.
[b] Osman *et al.*, 1999' Wang *et al.*, 2002; Wang *et al.*, 2002; Wilkinson *et al.*, 2002; Stebbing *et al.*, 2003a; Ribechini *et al.*, 2006
[c] Crowe *et al.*, 1991; Cannon *et al.*, 2003; Engels *et al.*, 2003; Mbulaiteye *et al.*, 2003
[d] Gao *et al.*, 1996; Kedes *et al.*, 1996; Lam *et al.*, 2002; Simpson *et al.*, 1996; Lin *et al.*, 1997

donors and in higher frequency from individuals with KS (Browning *et al.*, 1994). When isolated from the latter group, these spindle cells are infected with KSHV (Sirianni *et al.*, 1997). During HIV-1 infection, all arms of the immune system are affected. Suboptimal NK cell-, humoral- and cellular immune responses may therefore contribute towards the expansion of KSHV-infected EC to precipitate KS, and KSHV-infected plasmablasts to precipitate MCD or PEL.

Innate immunity

The innate arm of the immune system is the first host defence against invading pathogens. The interactions between KSHV and innate immunity are only starting to be explored.

Dendritic cells

Dendritic cells (DC) are professional antigen presenting cells with a pivotal role in the initiation of innate and adaptive immune responses. DC originate from CD34+ haematopoietic stem cells in the bone marrow and differentiate into the myeloid and lymphoid (or plasmacytoid) DC lineages (mDC and pDC respectively). mDC express myeloid lineage markers, remain in the circulation, or differentiate into immature DC (iDC) after migration to skin

(Langerhans cells, CD1a$^+$) and other tissues (interstitial DC, CD1a$^-$). mDC constantly sample their environment and upon foreign antigen encounter they migrate to the lymph nodes where they mature and present antigens to T lymphocytes. In parallel, pDC express lymphoid markers, remain in circulation or cluster around the high endothelial venules of inflamed lymph nodes. These cells migrate into the lymph nodes in response to inflammatory cytokines and they are considered to be the main type I interferon-producing cells during host defence against viral infections. In addition to these typical functions, DC exhibit an extraordinary plasticity and multi-potency in their ability to interact with all cells of the immune system to initiate and orchestrate efficient innate and adaptive immune responses (Banchereau and Steinman, 1998; Patterson, 2000; Shortman and Liu, 2002).

The key role of DC in host responses against viral infections makes them a major target for viral mechanisms to evade host immunity. The development of efficient in vitro iDC generation methods from peripheral blood monocytes (moDC) (Sallusto and Lanzavecchia, 1994) or bone marrow CD34$^+$ stem cells (Caux *et al.*, 1997) has allowed studies of the effects of viruses on DC generation, maturation, and function. Members of the herpesvirus family such as HSV, HHV6, CMV, and EBV use a variety of mechanisms to block DC generation and/or maturation, e.g., CMV directly infects DC and impairs their function and also blocks differentiation of monocytes to DC, whereas EBV induces apoptosis of monocyte DC progenitors (Chapters 31 and 43).

Currently, little is known about the interaction of DC and KSHV. In vitro, although infection of CD34$^+$ hematopoietic progenitors does not affect differentiation to DC (Larcher *et al.*, 2005), KSHV uses the DC-SIGN receptor to directly infect DC resulting in a decrease of their antigen presenting properties (Rappocciolo *et al.*, 2006). In vivo, the importance of DC in the host response to KSHV can be hypothesized based on the high occurrence of KSHV related malignancies in immunosuppressed individuals and the critical role that DC play in the pathogenesis of other herpesviral infections. Moreover, KS occurs at sites rich in DC: Langerhans cells in the skin and interstitial DC in the mucosal surfaces. In the context of HIV-1 infection, the number and function of both DC lineages decrease after primary infection and during high viraemia (Donaghy *et al.* 2001, 2003). A further reduction in the numbers of pDC has been reported in AIDS-KS in comparison to HIV-1 infected individuals (Stebbing *et al.*, 2003b). Low numbers of both DC subsets (but not CD34$^+$ hematopoietic progenitors) are also reported in classic KS and this decrease correlates with advanced disease (Della Bella *et al.*, 2006). Finally, the number of Langerhans cells is significantly decreased in KS lesions when compared with normal skin, suggesting that KSHV might influence DC maturation or migration (Valcuende-Cavero *et al.*, 1994). However, it is still not clear whether DC are infected by KSHV in vivo and the exact mechanisms that KSHV employs to effect DC maturation and function are yet to be determined.

Natural killer cells

Natural killer (NK) cells are lymphocytes that do not undergo genetic recombination events and thus do not express clonally distributed receptors for antigen. They are found mostly in peripheral blood, spleen, and bone marrow, but can migrate to inflamed tissues in response to various chemoattractants. NK cells mediate direct lysis of target tumor or infected cells by release of perforin and granzymes or by binding to apoptosis inducing receptors on the target cell. Upon activation, NK cells release inflammatory cytokines, which influence the type of adaptive responses. The mechanism of NK function is described by the "missing self" hypothesis (Medzhitov and Janeway, 2002): NK activity is switched off by a set of inhibitory receptors, which are expressed on their cell surface, including killer-cell immunoglobulin-like receptors (KIRs), immunoglobulin-like inhibitory receptors (ILT) and the lectin-like heterodimer CD94-NKG2A. All these receptors are involved in HLA molecule recognition. Each type of KIR is expressed only by a subgroup of NK cells allowing the constant sampling of every single HLA allele. Down-regulation of MHC-I molecules, which occurs in tumor and virally infected cells, results in the withdrawal of the KIR inhibitory signal and subsequent activation of NK cells through a group of receptors termed natural cytotoxicity receptors (NCR) (Medzhitov and Janeway, 2002; Moretta *et al.*, 2002). It has been shown that individuals with decreased or depleted NK activity are prone to HSV, CMV, and EBV infections (Biron *et al.*, 1999). Moreover, herpesviruses evade NK cell-mediated immunity using mechanisms that target every step of NK cell activation (Chapter 31 and Orange *et al.*, 2002).

At least four different mechanisms by which KSHV regulates NK activity can be proposed (for review see Orange *et al.*, 2002): Inhibition of NK activating receptor ICAM-1 by the viral gene K5, selective modulation of MHC-I molecules (HLA-A and HLA-B only) by K5, secretion of viral antagonists (vMIP-I and II) that block chemotactic responses of leukocytes, and decreased number of pDC (see DC section), which are the main type I IFN producers and fundamental for NK function. However, apart from the latter, these mechanisms involve lytic KSHV gene products and do not explain how KSHV latently infected cells that express low levels of MHC-I are protected from NK cells.

There is some evidence for the importance of NK cells in the control of KSHV infection and KS development: first, PEL cell lines are preferentially lyzed by NK cells from healthy blood donors when compared with KSHV$^-$/EBV$^+$ Burkitt lymphoma cell lines. Second, NK cell activity is decreased in individuals with aggressive AIDS-KS in comparison with individuals with indolent classic KS. Finally, NK cell activity is restored within 6 months of HAART in individuals with complete KS resolution and coincides with cell associated KSHV clearance (Sirianni *et al.*, 2002). However, it is still not clear whether NK cell activity is specifically driven by KSHV in infected individuals, as NK cell activity does not differ between HIV-1 infected individuals with and without KS. Further studies should investigate the role of NK cells in the control of KSHV infection and in the pathogenesis of KSHV-related neoplasms.

Complement

Complement activation occurs due to differences in pathogen envelope or membrane composition (alternative pathway), or existence of pathogen specific antibodies (classical pathway) (Walport, 2001a,b). Viruses have evolved evasion mechanisms of complement activity by incorporating complement regulatory proteins into their envelope or by having structural and functional or just functional viral homologues of such regulators (termed regulators of complement activation RCA). The importance of these regulatory homologues was demonstrated in a mouse model of γ-herpesvirus infection. Murine γ-herpesvirus 68 (MHV68) encodes for an RCA, which inhibits complement activation at the level of C3 (the point of convergence of all complement pathways). It was demonstrated that deletion of this protein resulted in decreased virulence and that this was reversed in C3$^-$/C3$^-$ mice. In addition, complement was shown to have a direct effect on viral latency (Kapadia *et al.*, 2002). Furthermore, and similarly to the MHV 68 KSHV encodes for a lytic product (ORF4) that inhibits complement activation at the level of C3 (named KSHV complement control protein, KCP) (Spiller *et al.*, 2003). Based on these observations, it seems reasonable to speculate that KCP plays an important role in the protection of KSHV virions and/or infected cells, against opsonisation, complement mediated virolysis, and humoral immune responses, in particular during cell-to-cell transmission.

Adaptive immunity

T-lymphocytes

T-lymphocytes are in the forefront of the battle of the host's immune system with invading pathogens. Activation of the innate arm of the immune system results in the direct killing of invading pathogens and the initiation and direction of efficient adaptive immune responses, which, if successful, lead to the establishment of immunological memory. Through the MHC machinery CD8+ and CD4+ T-lymphocytes specifically recognize viral peptide antigens, proliferate, and either directly lyze the infected cells (cytotoxic CD8+ T-lymphocytes, CTL) or further orchestrate the adaptive immune response (helper CD4+ T-lymphocytes, Th cells). However, during infection a variety of mechanisms are used by herpesviruses to escape T-cell responses (Ploegh, 1998; Yewdell and Hill, 2002).

Cytotoxic T-lymphocytes

CTL are primed by dendritic cells (DC) and by other professional antigen presenting cells, which present viral antigens through the MHC-I machinery. Following clonal expansion, the primed CTL act against virus by killing infected cells via perforin- and/or Fas-dependent pathways, before new virus particles are made, whereas they also release cytokines and chemokines with antiviral activity.

CTL epitopes

Considering the size of the KSHV genome, only a relatively small number of KSHV specific MHC class I-restricted CTL epitopes have been identified thus far (Fig. 52.1(a)) (Osman *et al.*, 1999; Wang *et al.*, 2000, 2001, 2002; Brander *et al.*, 2001; Micheletti *et al.*, 2002; Wilkinson *et al.*, 2002; Stebbing *et al.*, 2003a; Ribechini *et al.*, 2006). Although the use of these epitopes led to the detection of KSHV-specific CTL responses, these responses are in general weak compared to those seen against other herpesviral antigens. Whether this is due to viral immune escape from CTL, or whether help from autologous antigen presenting cells is necessary for efficient CTL responses (as shown for DC (Wang *et al.*, 2002)) or whether the KSHV genome has just not yet been exhaustively screened for CTL epitopes remains to be elucidated. One difficulty is that in the West, KSHV is predominantly present in HIV-1 infected individuals who exhibit suppressed CTL activity. The majority of CTL epitopes have therefore been identified in HIV-1 infected individuals during HAART, where the immune system is partly restored. Interestingly, T cell responses to LANA and ORF 65 are also detectable, even in KSHV-seronegative HIV-1 infected individuals, implying that KSHV-specific cellular immunity can occur in the absence of antibody responses (Woodberry *et al.*, 2005). However, the peptide epitopes present in these two viral proteins were not identified and it is not clear whether the observed responses were due to CTL or CD4$^+$ cells.

In KSHV, as in other herpesviruses, most of the CTL epitopes have been identified in conserved sites. It was demonstrated that functional CTL epitopes cluster in a

ORF (reference)	Protein product	Expression	Epitope (KSHV strain)	HLA I allele restriction
K1 (Stebbing*et al.*, 2003a)	Transmembrane glycoprotein	Lytic	FRLTERTLF (A) FRLTKTIFS (A) LRLTQQTFT (C) HRQSIWITW (A) HRQSIWHTL (C) YPQPVLQTL (C) YPQPVLQHA (A) YPQPVLQRA (A) QPVLQTLCA (A and B) QPVLQTLCG (C)	Cw*3 Cw*3 Cw*3 B*2702 B*2702 B*51 B*51 B*51 B*55 B*55
ORF 8 (Wang*et al.*, 2002)	Glycoprotein B	Lytic	LMWYELSKI	A*0201
ORF 22 (Micheletti *et al.*, 2002)	Glycoprotein H	Lytic	FLNWQNLLNV	A*0201
K8.1 (Wilkinson *et al.*, 2002)	Glycoprotein gp35-37	Lytic	ELTDALISAFSGSYS LILYLCVPRCRRKKP	A*0201 A*0201
ORF26 (Ribechini *et al.*, 2006)	ORF26	Lytic	FQWDSNTQL IVLESNGFDL VLDDLSMYL	A*0201 A*0201 A*0201
K3 (Ribechini *et al.*, 2006)	vMIR1	Lytic	LPRLTYQEGL GLAAATWVWL	B*7 A*0201
K5 (Ribechini *et al.*, 2006)	vMIR2	Lytic	ALYAANNTRV	A*0201
K12 (Brander *et al.*, 2001; Micheletti *et al.*, 2002; Ribechini *et al.*, 2006)	Kaposin A	Latent and induced in lytic	VLLNGWRWRL VVQELLWFL LYQRSGDMGL SYSLLTYML YMLAHVTGL TPRPFPRLEI	A*0201 A*0201 A*24 A*24 A*0201 B*7

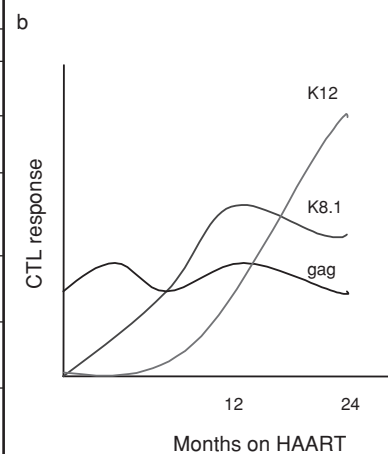


Fig. 52.1. KSHV specific CTL epitopes and CTL recovery during HAART. **(a)** KSHV specific CTL epitopes: CTL peptide epitopes have been identified in five KSHV encoded proteins. The K1 epitopes all cluster in the most variable region (VR1) and are associated with specific KSHV strains (associated strains are shown in brackets next to the epitopes). No HLA class A epitopes have thus far been identified in K1. K12 (Kaposin A) is the only latent KSHV protein thus far shown to elicit CTL responses. **(b)** Effects of HAART on the recovery of anti-KSHV CTL responses: CTL activity increases during HAART in HIV-1 infected individuals, with or without KS, against lytic and latent epitopes (K8.1 and K12, epitopes, respectively. HIV-1 gag responses also shown). CTL responses against K12 continue to increase after 12 months on HAART (Bourboulia *et al.*, 2004). These CTL responses could contribute to the observed drop in the incidence of KS in the West since the widespread introduction of HAART. CTL, cytotoxic T-lymphocyte; HAART, highly active anti-retroviral therapy.

positively selected region of the most variable KSHV gene. K1 is a positional homologue of LMP-1 of EBV and contains the two most variable regions (VR1 and VR2) across the entire KSHV genome which are used to classify KSHV into four clades (A, B, C, and D) (Zong *et al.*, 1999). Every viral isolate studied thus far is unique to an infected individual. However, unlike the situation in retroviruses, K1 mutations have not been detected within an infected individual over time. Several, HLA class I restricted epitopes within the VR1 were identified with the use of autologous overlapping peptides corresponding exactly to a patient's own viral sequence (Fig. 52.1(a)). These CTL epitopes are conserved within a specific strain (e.g. A or B), but not between strains. Based on these observations it appears that part of the genetic variability occurring in K1 is driven by a positive selection for CTL recognition, rather than due to CTL escape (Stebbing *et al.*, 2003a). Furthermore, the observed variability does not appear to be due to escape from humoral immunity (a mechanism employed by other viruses such as influenza and HIV-1 where variability in viral surface proteins is driven by escape from humoral immunity resulting a 'antigenic shift'). It seems likely that this selection prevents the complete evasion of all host immune control mechanisms, which would lead to overwhelming viral infection with subsequent death of the host and, therefore, of the virus. It seems that K1, which is an

early-lytic product, serves as a "suicide" protein allowing CTL recognition of cells reactivating from latency. Of note, CTL epitopes were also identified in vMIR1 and vMIR2 (Ribechini *et al.*, 2006), two lytic KSHV genes that down-regulate expression of MHC-I and other co-stimulatory proteins (Chapters 31 and 62), whereas vFLIP is able to upregulate expression of MHC-I and ICAM-1 (Lagos *et al.*, unpublihsed data). In addition to K1 recognition by CTL, the above findings reveal two more mechanisms employed by KSHV to regulate immune escape, limit viral dissemination and establish equilibrium in the virus–host co-speciation.

Effects of HAART on CTL

HAART promotes long-term immune reconstitution in patients with and without KS. This reconstitution is also KSHV-specific (Wilkinson *et al.*, 2002; Bourboulia *et al.*, 2004). It has been demonstrated that there is a significant decline of KSHV DNA load in PBMC and plasma during HAART and this correlates with a significant increase of anti-KSHV-specific CD8+ T-cell responses (Bourboulia *et al.*, 2004) (Fig. 52.1(b)). It appears that prolonged HAART (more than 12 months) is necessary for these anti-KSHV effects to be established and maintained in most HIV-1 infected individuals. In individuals with KS, resolution of KS is also associated with significant increases of KSHV specific CD8+ T-cell responses during the first 6–9 months on HAART. Future work has to determine whether other KSHV specific epitopes are able to elicit more potent CD8+ T cell responses and contribute to the restoration of KSHV specific T-cells and whether such responses are stronger and occur earlier than those observed for K8.1 and K12. Moreover, detailed phenotyping of KSHV-specific CD8+ T-cells would give further insight into the anti-KSHV responses in comparison to other viruses that establish persistent infections (Appay *et al.*, 2002).

Helper T-lymphocytes

The final stages of herpesvirus virion assembly occur in endosomal cellular compartments with extensive targeting of viral proteins to endosomes. During this process viral proteins can be efficiently sampled by the MHC-II, leading to the presentation of viral antigens to CD4+ T-lymphocytes.

The association of KS with low CD4+ T-cell count (mainly below 200/mm^3; although on average these values are higher than those associated with other AIDS-associated cancers) (Crowe *et al.*, 1991; Cannon *et al.*, 2003; Engels *et al.*, 2003; Mbulaiteye *et al.*, 2003) and the rapid KS resolution during HAART suggest a potential role for anti-KSHV CD4+ T-cell responses in the control of KSHV infection. However, although CD4+ T-cell proliferation as a response to KSHV has been reported (Strickler *et al.*, 1999), a correlation between CD4+ T-cell number and KS resolution during HAART has not been observed (Gill *et al.*, 2002). Future studies should determine the KSHV specific CD4+ T-cell epitopes and the patterns of these responses during primary and persistent infection comparing also with other herpesviruses (Amyes *et al.*, 2003).

B-lymphocytes

Antibody responses from B lymphocytes play a major role in anti-viral immunity (Burton, 2002). Antibodies can bind to viral proteins and block viral entry to the host cells (neutralizing antibodies), inhibit release of viral particles from infected cells, or trigger effector functions through their Fc domain causing the lysis of free virions or infected cells by NK cells (antibody-dependent cellular cytotoxicity, ADCC) or by the complement (complement dependent cytotoxicity, CDC).

The importance of humoral immune responses against human herpesviruses has been shown (Chapters 34, 43, 51 and 72). Furthermore, antibody responses against MHV68, in the absence of CD8+ and CD4+ T-cells, can efficiently control MHV 68 replication (Kim *et al.*, 2002).

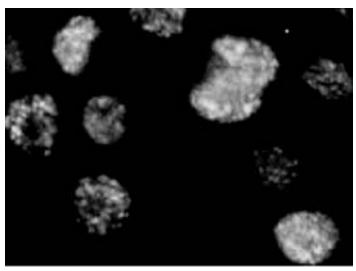
Antibody responses are recognized and detectable against KSHV latent and lytic proteins. However, the role of these antibodies in controlling KSHV replication or infection remains to be elucidated.

Antibody epitopes and serological assays for anti-KSHV antibody detection

The ORF73 of KSHV encodes for the major latent nuclear antigen of KSHV. More than 70% of infected individuals have detectable anti-LANA antibodies (Gao *et al.*, 1996; Kedes *et al.*, 1996). These humoral immune responses are directed against epitopes in the C-terminus. Detection of anti-LANA antibodies correlates in different populations with the KS burden (Chapter 54), and the detection of anti-LANA antibodies by indirect immunofluorescence is a useful assay for serological surveys (Fig. 52.2(a)). Several studies have suggested that seroconversion and/or a high antibody titer against LANA correlates with risk of KS development in HIV-1 infected individuals (Gao *et al.*, 1996; Sitas *et al.*, 1999; Sitas and Newton, 2001; Ziegler *et al.*, 2003). However, the association between KSHV load in sera or in PBMC and anti-LANA antibody titer is still unclear.

Strong anti-lytic antibody responses are directed against K8.1, which encodes for an envelope glycoprotein that is the positional homologue of EBV gp220/350 (Chandran *et al.*, 1998). Anti-K8.1 antibodies are detectable in approximately 80% of individuals with AIDS-KS and in more than 90% of

(a)



(b)

Assay	Sensitivity	Specificity
Lytic IFA	>95	80-85
LANA IFA	70-90	>95
ORF65 IFA	40	>95
Viral lysate ELISA	85-95	85-90
Recombinant LANA ELISA	60-70	85-90
Recombinant ORF65 ELISA	75-85	85-90
ORF65 peptide epitope ELISA	80-90	85-90
Recombinant K8.1 ELISA	80-95	90-95
K8.1 epitope ELISA	75-95	90-95
MAP K8.1 ELISA	80-95	>95
K8.1 and ORF65 peptide epitope mix ELISA	85-95	90-95
Recombinant K12 ELISA	55	90

Fig. 52.2. Humoral responses to KSHV and reconstitution during HAART (a) Immunofluorescent assay for anti-LANA antibodies: The KSHV LANA elicits antibody responses and is used for serological studies in an indirect IFA. Sera from KSHV infected individuals recognize LANA and a typical nuclear stippling pattern is observed. **(b)** Several KSHV proteins have been shown to elicit potent humoral responses and used in the development of serological assays for the detection of KSHV infection: Sensitivity and specificity of various serological assays as described in comparative studies (see main text) or unpublished results. Sensitivity is calculated based on the assumption that all individuals with KS should be positive for KSHV. In general, assays combining different proteins (e.g. ELISA combining K8.1 and ORF65 epitopes) are more sensitive than single assays. **(c)** The detection of anti-KSHV antibodies in different populations correlated with KS burden: Various serological assays have been employed to conduct KSHV epidemiological. Although the sensitivity and specificity of these assays vary, overall the global seroprevalence correlated with the incidence of KS: White bars correspond to countries with a low KS incidence (mainly AIDS-KS). Light gray to Mediterranean countries where classic KS occurs, and dark grey to Sub-Saharan Africa with a high prevalence of non-HIV-1 (endemic-) and AIDS-KS, West Africa remains a conudrum, because KS is seldom seen, despite a high seroprevalence of KSHV (reviewed in Ablashi *et al.*, 1999; Sarid *et al.*, 1999; Dedicoat and Newton 2002; Dukers and Rezza 2003; Martin 2003; figure courtesy of Dimitra Bourboulia). (d) The effect of HAART on anti-KSHV humoral responses: LANA and K8.1 epitopes have been used to assess the effect of HAART on KSHV specific humoral responses (Bourboulia *et al.*, 2004). Reconstitution of humoral immunity during HAART, and in particular an increase number of individuals with detectable anti-K8.1 antibodies, coincides with a reduction of plasma KSHV viraemia. However, the role of these humoral responses in controlling KSHV replication is still not elucidated. LANA latent nuclear antigen; IFA, immunofluorescence; ORF, open reading frame, ELISA, enzyme linked immunosorbent assay; MAP, multiple antigenic peptide

individuals with non-AIDS KS (Fig. 52.2(b)). A K8.1 peptide epitope, with no known sequence similarity to any other pathogen has been used in the form of a multiple antigenic peptide (MAP) for the development of a highly sensitive and specific ELISA to detect KSHV infection (Lam *et al.*, 2002). The sensitivity of this ELISA is improved by combining the K8.1 epitope with a known LANA epitope in a MAP (D.Lagos, unpublished data).

Another lytic antigen that generates humoral responses is the small viral capsid antigen encoded by ORF65 (Simpson *et al.*, 1996; Lin *et al.*, 1997). A combination of an ORF65 peptide epitope with a K8.1 epitope is employed in one of the most sensitive and specific, commercially available assays (Schatz *et al.*, 2001) (Fig. 52.2(b)).

Specific antibody responses among individuals with KS have also been reported against ORF26 and K12, although

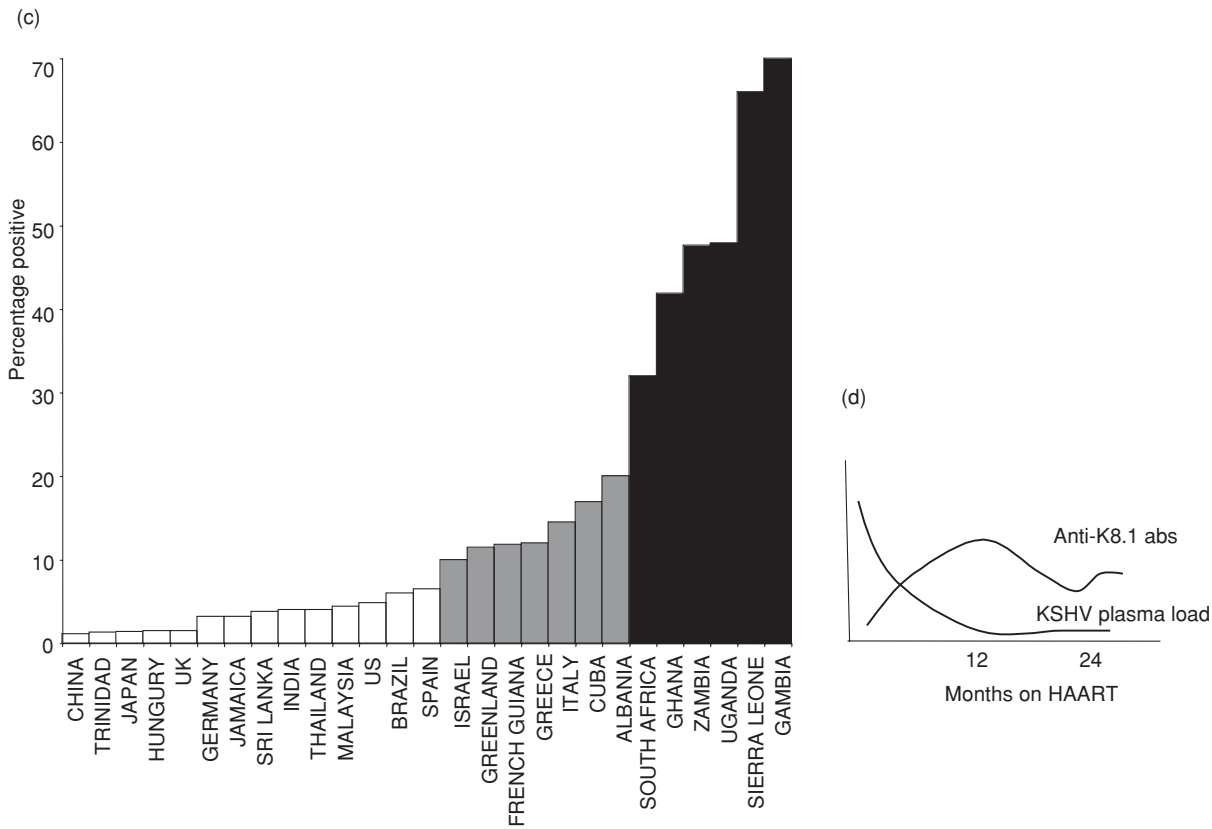


Fig. 52.2. (*cont.*)

significantly less common than LANA, K8.1, and ORF65 (Schatz *et al.*, 2001).

A variety of serological assays for KSHV detection have been described. These include immunofluorescent assays using PEL cell lines and ELISA using virions, purified recombinant proteins, peptide epitopes, mixtures of peptide epitopes, and multiple antigenic peptide epitopes as antigens. These assays have been successfully used in large epidemiological studies providing insight into KSHV biology, transmission, risk factors for KSHV infection and KS development (Ablashi *et al.*, 1999; Sarid *et al.*, 1999; Dedicoat and Newton, 2002; Dukers and Rezza, 2003; Martin, 2003) (Fig. 52.2(c)). The sensitivity of these assays ranges from less than 80% for individuals with AIDS-KS to more than 90% for HIV-1 negative individuals with KS (Rabkin *et al.*, 1998; Enbom *et al.*, 2000; Spira *et al.*, 2000; Schatz *et al.*, 2001; Dukers and Rezza, 2003; Pellett *et al.*, 2003) (Fig. 52.2(b)). There are some AIDS-KS cases where anti-latent and anti-lytic antibodies are not detectable, but KSHV copies can be detected in plasma and/or PBMCs by quantitative PCR (Lallemand *et al.*, 2000). Whether this is due to low sensitivity of the current serological assays or the defect in the humoral immunity of these individuals remains to be elucidated.

Effects of HAART on humoral responses

Although it is not clear if HAART has any significant effect on anti-LANA antibody titer (Wilkinson *et al.*, 2002; Bourboulia *et al.*, 2004), an increase in the number of individuals with detectable anti-K8.1 antibodies coincides with plasma virus clearance during HAART (Bourboulia *et al.*, 2004) (Fig. 52.2(d)). Based on these results it could be proposed that the humoral arm of the immune system plays an important role in the control of the KSHV replication.

How antibodies contribute to KSHV immunity

It has been shown that sera from KSHV-seropositive individuals can block KSHV infection in vitro (Dialyna *et al.*, 2004; Kimball *et al.*, 2004). Moreover, individuals with AIDS-KS display reduced levels of neutralizing antibodies,

Box 52.2. Transmission of cancer, immunity and Kaposi's sarcoma

Until the end of the eighteenth century, it was believed by many that cancer was a contagious disease. James Nooth, an English surgeon, and Jean Louise Alibert, the founder of the French School of Dermatology and personal physician of King Louis XVIII, were the first to independently challenge this hypothesis: both injected themselves with breast cancer cells, which resulted in short-lived local inflammatory responses, but no tumour establishment. They concluded that cancer was not contagious.

The discovery in 1908 by Peyton Rous that cell-free filtrates from tumors could transmit cancer between Plymouth Rock hens heralded the era of viral oncology and sparked a debate whether viruses were responsible for most cancers. The first evidence that a virus was indeed involved in the pathogenesis of a human cancer only emerged in 1964 with the discovery of Epstein–Barr virus. Two years later Peyton Rous was awarded the Nobel Prize in Physiology of Medicine for his discovery of "tumor inducing viruses."

In the 1950s, selective immunosuppressant drugs were developed and organ transplantation became a life-saving reality. However, the first case of a kidney transplant recipient who developed iatrogenic Kaposi's sarcoma was reported soon afterwards. It was also noticed that Kaposi's sarcoma can disappear when immunosuppression is stopped, linking immunity closely with Kaposi's sarcomagenesis. The transmission of other cancers during organ transplantation, e.g., melanoma, has also been documented.

Until recently, it was widely accepted that iatrogenic Kaposi's sarcoma was due to the loss of immunesurveillance against KSHV: It was thought that KSHV could be transmitted from donor to host, or that immunosuppressive drugs led to the reactivation of KSHV in already infected recipients. The provocative finding in 2003 that certain cases of post-organ transplant Kaposi's sarcoma are due to the transmission of KSHV-infected precancerous cells, revived the debate on the transmission of cancer. Ironically, this is a tumor induced by a virus, where both host cell and pathogen are transmitted simultaneously. These findings infer that KSHV not only infect endothelial cells during periods of immunodeficiency, but that KSHV-infected circulating endothelial cells are present in otherwise healthy individuals. Notably, the concept of cancer cell transmission is further supported by the identification in 2006 of the oldest known somatic mammalian clonal cell population as the causative agent of the canine transmissible venereal tumor.

The wider implications of this are that in populations such as certain sub-Saharan African countries with a large HIV-1 burden where many are immunosuppressed, the transmission of cancer cells between infected individuals, e.g., Kaposi's sarcoma or anogenital cancer may represent a reality. The study of iatrogenic Kaposi's sarcoma would provide further insight into the immunological control of cancer.

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despite the fact that higher total binding antibody levels are observed in this group (Kimball *et al.*, 2004). This implies that the humoral immunity plays a crucial role in the prevention of KS development and that the most immunogenic KSHV antigens are not necessarily the targets of neutralizing antibodies. However, the contribution of KSHV neutralizing antibodies in controlling in vivo infection needs to be addressed further, as the neutralizing titers are low and the specific epitopes not known. K8.1 could be such a neutralizing antibody target as it is directly involved in the process of viral entry into host cells by binding to heparan sulfate (Birkmann *et al.*, 2001; Chapter 23) and reconstitution of anti-K8.1 humoral responses during HAART coincides with virus clearance. Of note, gp 220/350, the EBV positional homologue of K8.1, elicits neutralizing antibody responses and has been investigated as a target for development of an EBV vaccine (Chapter 72). Another candidate for KSHV-specific neutralizing antibodies is glycoprotein B, which is also involved in viral entry (Chapter 23). Rabbit polyclonal antibodies raised against glycoprotein B

neutralize infectivity in vitro (Akula *et al.*, 2002). In addition to their neutralizing potential, antibodies can control KSHV infection through their effector functions. It has been reported that the genotype of the low affinity Fc receptor FcγRIIIA can contribute protection or conversely be a risk factor for KS development (Lehrnbecher *et al.*, 2000). This receptor is expressed on the surface of NK cells and could be an important factor influencing ADCC activity. Further studies are necessary to elucidate the role of the humoral immunity in the host defense against KSHV.

Conclusions and future perspectives

Excluding pox viruses, KSHV has pilfered an unprecedented array of cellular genes, mainly to impede the function of host antiviral immune responses. During immunosuppression, both the innate and adaptive anti-KSHV immune responses are hampered, allowing the uncontrolled proliferation of KSHV-infected cells that belong to the B-lymphocyte and EC lineages.

KSHV proteins that elicit humoral and cellular immune responses are being identified and the consequences of immunodeficiency (HIV-1 induced or iatrogenic) and immune-restoration are being elucidated. Such studies will allow the understanding of the anti-KSHV host defence mechanisms and could eventually lead to the generation of an effective vaccine. During the next couple of years, the study of KSHV immunobiology should also provide further insight into tumor and transplant immunology.

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The epidemiology of EBV and its association with malignant disease

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EPSTEIN-BARR VIRUS EPIDEMIOLOGY

Epidemiology of primary Epstein-Barr virus infection

Epstein-Barr virus (EBV) is an ancient virus, and has probably coevolved with its different hosts over the last 90–100 million years (McGeoch *et al.*, 1995). With the ability to establish lifelong latency and intermittent reactivation after primary infection and with limited clinical symptoms in the majority of infected individuals, EBV has become ubiquitous in all human populations

Age at primary infection

Children in developing countries acquire the infection in the first few years of life, and universal seroconversion is often seen by ages 3–4 years, whereas infection in developed countries often is delayed until adolescence (de The *et al.*, 1975; Haahr *et al.*, 2004; Henle and Henle, 1967; Melbye *et al.*, 1984a,b) (Figure 53.1). In some developed countries a bimodal infection rate, with peaks in children below 5 years and again after 10 years of age, has been described (Edwards and Woodroof, 1979; Henle and Henle, 1967; Lai *et al.*, 1975). Oral EBV excretion between parents and infants, and from intimate partners in adolescence and early adulthood is the likely explanation for the observed bimodality (Crawford *et al.*, 2002; Fleisher *et al.*, 1979).

EBV antibody titers in seropositive individuals vary according to age following a U-shaped pattern, with high titers among infants and in the elderly (above 50 years) (Glaser *et al.*, 1985; Venkitaraman *et al.*, 1985). High antibody titers in infants probably reflect primary infection, whereas in the elderly it may be due to an age-dependent reactivation due to a reduced cellular immune response (Wick and Grubeck-Loebenstein, 1997).

Geographic variation

EBV has been detected in all populations and all areas of the world (IARC, 1997), but with noticeable geographical variation in the distribution of EBV genotypes. Two major types of EBV, type 1 and 2, have been described in humans, varying in the genes that encode some of the nuclear proteins in latently infected cells (Sample *et al.*, 1990). Both types are detected all over the world, with type 1 being the most prevalent. However, in some regions (e.g. central Africa, Papua New Guinea and Alaska) type 2 is more prevalent (Table 53.1) (Gratama and Ernberg, 1995; Zimber *et al.*, 1986). It is assumed that the geographical distribution of the two types in EBV-associated diseases reflects the general prevalence in the areas involved (Gratama and Ernberg, 1995). Thus, there seems to be no clear association between the two types and specific diseases.

In most areas recovery of type 2 is unusual, except in immunodeficient (HIV+ individuals and transplant recipients) carriers. The increased detection in immunodeficent individuals has been explained by an increased exposure to exogenous virus combined with deficient EBV-specific cellular immunity, leading to long-term carriage of multiple EBV genotypes (Gratama and Ernberg, 1995). The frequency of the type 2 genotype in HIV-positive haemophiliacs is comparable to the frequency in healthy individuals, which indicates that the immunodefiency *per se* is not responsible for increased type 2 detection (Yao *et al.*, 1998).

The distribution of specific DNA sequence polymorphisms also shows geographic variation, with the epidemiology of the EBV-encoded oncogene LMP-1 being the most thoroughly studied. Numerous sequence variations have been identified in LMP-1 genes from different EBV isolates, some of which have been associated with an increased risk of nasopharyngeal carcinoma (Jeng *et al.*, 1994).

Table 53.1. Distribution of EBV types 1 and 2 in various clinical conditions and among healthy patients. (Partly reproduced from Gratama and Ernberg, 1995 with permission from Elsevier.)

Country	Patients	N	Type 1 (%)	Type 2 (%)	Both types (%)
China (Hu *et al.*, 1991)	Nasopharyngeal carcinoma	37	86	14	0
Taiwan (Shu *et al.*, 1992)	Nasopharyngeal carcinoma	53	94	4	2
Korea (Kim *et al.*, 2002)	Healthy	26	81	15	4
Japan (Kunimoto *et al.*, 1992)	Healthy	21	95	5	0
USA (Frank *et al.*, 1995)	Post-transplant lymphoproliferative disease	24	100	0	0
USA (Goldschmidts *et al.*, 1992)	HIV-positive	22	55	45	0
Alaska (Abdel-Hamid *et al.*, 1992)	Nasopharyngeal carcinoma	3	0	100	0
Argentina (Correa *et al.*, 2004)	Healthy	183	78	15	7
Brasil (Klumb *et al.*, 2004)	Burkitt's lymphoma	21	86	14	0
Central Africa (Goldschmidts *et al.*, 1992)	Burkitt's lymphoma	16	50	50	0
Papua New Guinea (Aitken *et al.*, 1994)	Burkitt's lymphoma	56	42	53	5
Western Europe (Sandvej *et al.*, 1994)	Hodgkin's disease	55	93	5	1
Australia (Kyaw *et al.*, 1992)	HIV-positive	56	27	30	43
Australia (Kyaw *et al.*, 1992)	Cardiac transplants	18	39	33	28

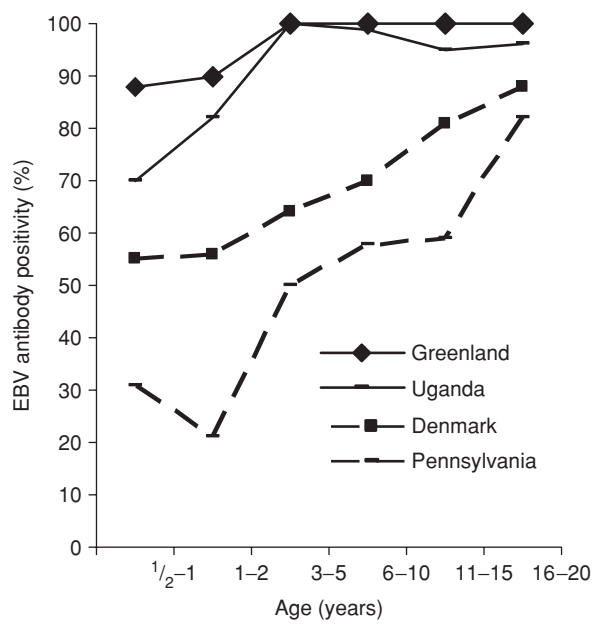

Fig. 53.1. Age-specific distribution of EBV antibody positive individuals in four populations. Reproduced from de The *et al.*, 1975; Henle and Henle, 1967; Melbye *et al.*, 1984.

Studies indicate that LMP-1 sequence variants from nasopharyngeal carcinoma high-incidence areas in Southeast Asia have evolved distinct from LMP-1 variants in nasopharyngeal carcinoma low-incidence areas such as areas of Australia, Papua New Guinea, and Africa (Burrows *et al.*, 2004) suggesting that positive selection pressure on the LMP-1 sequences may enhance the oncogenic potential of virus isolates from nasopharyngeal carcinoma endemic areas.

Sex differences

There is no consistent difference in EBV seroprevalence by sex in children (Golubjatnikov 1973; Lang *et al.*, 1977; Sumaya *et al.*, 1975). In developed countries, this similarity continues into early adolescence, when a higher seroprevalence and an earlier occurrence of infectious mononucleosis among girls indicate earlier exposure to the virus (Crawford *et al.*, 2002).

Generally, antibody titers seem to be higher in females than in males (Biggar *et al.*, 1981; Levine *et al.*, 1982; Wagner *et al.*, 1994). This difference, which also has been observed for other viruses, is in accordance with the notion that women in general mount more vigorous antibody- and cell-mediated immune response following infection or vaccination than men (Beagley and Gockel, 2003).

Socioeconomic factors

Poor socioeconomic conditions have been associated with early primary EBV infection, whereas late primary EBV infection is seen in populations of high socioeconomic status. Based on father's education and the family's living conditions, Henle and colleagues documented a seroprevalence of 60% among young American school children (aged 5 to 10 years) of low socioeconomic status compared to less than 20% in children with high socioeconomic level (Henle

et al., 1969). Low income and crowded family conditions have also been found to increase the likelihood of being EBV seropositive in children from other geographical locales, such as Thailand (Mekmullica *et al.*, 2003), Turkey (Ozkan *et al.*, 2003), Ghana (Biggar *et al.*, 1978) and Denmark (Hesse *et al.*, 1983). In an early work by Lang and colleagues (Lang *et al.*, 1977) three genetically different Melanesian populations with differences in living conditions and social patterns were found to have similar patterns of early EBV infection. In all three populations the mothers chew the food before feeding the children. Thus, exposure to saliva either directly or via for example unclean toys are believed to explain differences in socioeconomic conditions. This is in line with the observation that socioeconomic associated differences in sero-prevalence are greatly diminished in age groups who have become sexually active.

Genetic and racial factors

Differences in prevalence and more generally the infection patterns have never been clearly associated with race, but merely seen as differences in socioeconomic, hygienic, and cultural behavior. The high prevalence of EBV infection in populations around the world would indicate that the influence of host-specific characteristics on natural resistance to EBV infection is limited. Yet, the distinct distribution of nasopharyngeal carcinoma (NPC) with high incidence figures observed in Inuits from the Arctic and in South-East Asian populations suggests the existence of a particular immunologic control of EBV in these ethnicities, though the exact mechanism remains to be described (Hildesheim *et al.*, 2002; Yu and Yuan, 2002). Supportive of a genetically determined response to the EBV carrier state is the finding among Greenlandic Inuits of remarkably high titers throughout life of IgG antibodies to EBV-VCA (Melbye *et al.*, 1984a,b).

Transmission

Most frequently, EBV transmission takes place through oropharyngeal secretion. In adolescent and adult cases of IM intimate kissing has been the main route of transmission whereas saliva on, for example, toys and fingers is believed to be major routes of transmission among smaller children. However, 40 years into the discovery of EBV we still need data to better explain the exact determinants of infection. Among EBV seronegative adults, close contact with IM cases (Sawyer *et al.*, 1971), or longer stays together with seropositive persons in a restricted space (Storrie and Sphar, 1976) only infrequently leads to secondary cases. On the contrary, EBV infection frequently takes place among smaller children of low socioeconomic status, in nurseries (Chang *et al.*, 1981) and when sharing a room (Crawford *et al.*, 2002).

Shedding of EBV in saliva among seropositive individuals ranges from 22% to 90% (Apolloni and Sculley, 1994; Haque and Crawford, 1997; Ikuta *et al.*, 2000; Sixbey *et al.*, 1984;Yao *et al.*, 1991). Ling and colleagues observed that shedding of EBV in saliva among adults at any given time over a 12 months period varies between 32% and 73% (Ling *et al.*, 2003). These and other authors were unable to detect any correlation between viral shedding frequency or viral load in saliva and the presence of EBV in PBMCs (Haque and Crawford, 1997; Ling *et al.*, 2003), suggesting that the factors responsible for EBV reactivation in the oropharynx are different from those governing viral load in the blood.

EBV has been detected in cervical secretions in between 8 and 28% of teenage girls and adult women, and in semen samples and samples scraped from the penile sulcus of men (Enbom *et al.*, 2001; Israele *et al.*, 1991; Kapranos *et al.*, 2003; Naher *et al.*, 1992), but evidence on whether EBV is transmitted through genital contact is limited. A recent study found EBV type 2 among homosexual men to be significantly more prevalent than among heterosexual men and to be correlated with the number of sexual partners (van B. D. *et al.*, 2000). However, the exact mode of transmission in these studies remains unknown since it is difficult to distinguish between genital transmission, orogenital contact and kissing.

Transplacental transmission and transmission through breast milk have been reported in rare circumstances, but are considered non-significant modes of transmission (Fleisher and Bologonese, 1984; Kusuhara *et al.*, 1997; Meyohas *et al.*, 1996). EBV may be spread through blood transfusion and as a result of organ transplantation (Alfieri *et al.*, 1996; Scheenstra *et al.*, 2004). One transfusion unit of erythrocytes contains an average of two EBV genomes, in contrast to a whole blood unit which harbored on average 600 to 700 EBV genomes (Wagner *et al.*, 1995). Transmission is of particular concern in association with organ transplantations where primary EBV infection is a major risk factor for post-transplant lymphoproliferative disease (PTLD) (Aguilar *et al.*, 1999; Bodeus *et al.*, 1999; Scheenstra *et al.*, 2004).

EBV viral load epidemiology

During the recent decade, methods for detecting and quantifying cellular and extracellular EBV in peripheral blood have improved significantly. Initially applicable only to small series of patients, modern techniques such as

real-time PCR have now made large studies feasible. However, the majority of these have been performed to investigate EBV viral loads in specific diseases, and knowledge on EBV viral load in healthy individuals has mainly been generated from the control groups, rarely selected randomly from the population.

EBV viral load in peripheral blood mononuclear cells (PBMCs) is the combined result of the number of infected B cells and the number of EBV genomes per B-cell. A roughly constant number of infected B cells (1–50 *per* 1,000,000) is present in peripheral blood in the healthy latently infected host, but the number seems to vary considerably between individuals (Khan *et al.*, 1996). Differences in detection methods, sample preparation and measurement units make comparisons of EBV viral loads from different studies difficult. But the EBV viral load appears to be transiently elevated at the time of primary EBV infection (Fan and Gulley, 2001). In general, the viral load observed in PBMCs from healthy individuals is low (<100 DNA genome copies *per* ug DNA) compared to the high EBV loads observed in some EBV-associated diseases (ex. post-transplant lymphoproliferative disease (PTLD)) (Stevens *et al.*, 2002a,b). The low EBV viral loads in most healthy EBV-infected individuals reflect the low frequency of EBV-positive B cells in the circulation, whereas the high EBV loads observed during PTLD and immune suppression are the result of increased numbers of EBV infected B cells (Babcock *et al.*, 1999; Yang *et al.*, 2000), together with an increased number of EBV genomes in some of the infected B cells (Rose *et al.*, 2002). However, EBV viral load in PBMCs in the single healthy individual does not appear to be static. As healthy individuals are followed over time, short episodes of increased viral load can be observed, suggestive of EBV reactivation (Maurmann *et al.*, 2003), and measurement of EBV viral load in PBMCs and plasma seem to detect episodes of EBV reactivation earlier and with greater sensitivity than the traditional serological methods (Figure 53.2). EBV detection in whole blood and serum/plasma is becoming the 'gold standard' and most commonly utilized test, as it is less laborious and more reproducible than PBMCs.

The correlation between EBV viral load in PBMCs and serological response is not obvious (Gartner *et al.*, 2000). Increased anti-p18-VCA, suggestive of lytic viral replication, and decreased anti-EBNA-1 IgG levels has been associated with high EBV loads in HIV carriers (Stevens *et al.*, 2002a,b).

EBV in plasma or serum, which is frequently detected in patients with nasopharyngeal carcinoma or PTLD, is only rarely detected in healthy individuals, although EBV viremia can be detected in association to episodes of EBV reactivation (Lechowicz *et al.*, 2002;).

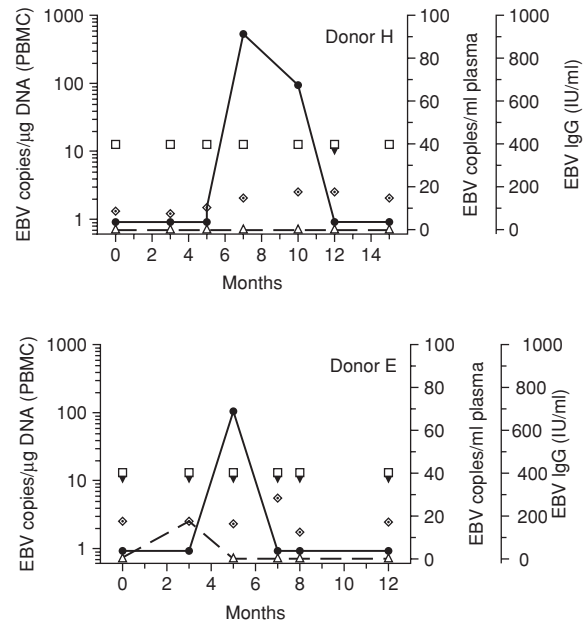

Fig. 53.2. Healthy individuals with serological evidence of EBV reactivation during 15 months of follow-up. -_- viral load (PBMC), -△- Viremia (plasma), ◇ EBV IgG (IU/ml). Partly reproduced from Maurmann *et al.*, 2003.

Infectious mononucleosis

Primary EBV infection is usually considered asymptomatic when occurring in infants and small children, and most of the knowledge on primary infection is derived from studies of adolescent patients with infectious mononucleosis. However, the assumption that primary EBV infection in childhood is always subclinical is probably fallacious, as when looked for, infectious mononucleosis symptoms may also occur in infants in association with primary EBV infection (Chan *et al.*, 2003). Although symptoms may be milder, primary infection in infants is not presumed to be fundamentally different from the characteristic picture of infectious mononucleosis (IARC, 1997).

Primary EBV infection in adolescence causes infectious mononucleosis in more than half of the infected individuals. Symptoms of infectious mononucleosis (glandular fever) commence after an incubation period of 4–7 weeks, and typically (in more than 50%) include fever, lymphadenopathy and pharyngitis (Chang, 1980).

Confirmation of the diagnosis has traditionally been based on the detection of heterophil antibodies, which are present in 86% of adolescents and adults with infectious mononucleosis (Fleisher *et al.*, 1983), but less frequently in acutely infected small children (Chan *et al.*, 1998). However,

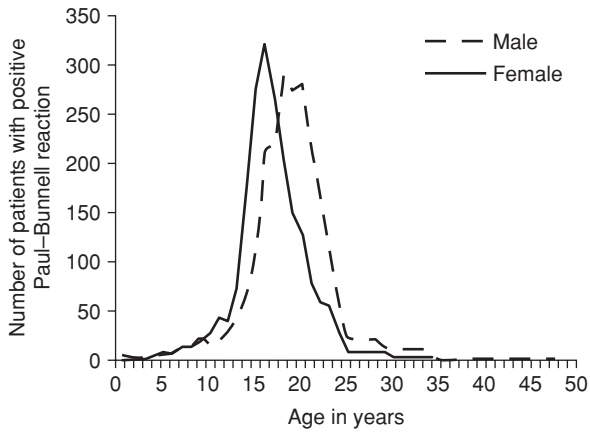

Fig. 53.3. Age-and sex-specific distribution of positive Paul–Bunnell reactions at Statens Serum Institut in Denmark 1965–1969. Reproduced from Rosdahl *et al.*, 1973 with permission from Taylor & Francis Scandinavia.

positive detection of heterophil antibodies can occur in other conditions, including HIV infection, Systemic Lupus Erythematosus, and other viral diseases (Hendry and Longmore, 1993; Horwitz *et al.*, 1979; Macsween and Crawford 2003). Detection of IgM to the viral capsid antigen (VCA) is both more sensitive and specific, and is present at time of onset of clinical symptoms. Both heterophil antibodies and anti-VCA IgM are transient and disappears within months, and detection of EBV-DNA in serum might be useful as a supplement to serology for the diagnosis (Chan *et al.*, 2001).

Epidemiology

The majority of infectious mononucleosis patients are not hospitalized, but reliable data on the population incidence is available from sentinel systems, centralized laboratories or from areas where infectious mononucleosis is a reportable disease. Incidence rates between 60–100 *per* 100,000 person-years in Caucasian populations seem consistent (Evans *et al.*, 1997; Morris and Edmunds, 2002; Rosdahl *et al.*, 1973). In these populations the incidence of infectious mononucleosis increases from the age of 2–4 years to reach a maximum in adolescence and early adulthood, after which the disease incidence decreases, to become rare after 40 years of age (Figure 53.3) (Auwaerter, 1999; Rosdahl *et al.*, 1973). The age-specific distribution of cases reflects the clinical disease ratio of primary EBV infection which is low in children, and may reach 74% among college students (Sawyer *et al.*, 1971). The low incidence among older adults reflects the low number of EBV-uninfected individuals. There seems to be no seasonal variation in incidence rates.

Table 53.2. Factors influencing the development of infectious mononucleosis

EBV immune status
Age at time of infection
IL-10 polymorphisms

The difference in infectious mononucleosis incidence rates between ethnic groups within the same region, probably reflects variation in social and economic factors, influencing age at primary infection (Heath *et al.*, 1972; Melbye *et al.*, 1984a,b), and there is no evidence of racial differences in infectious mononucleosis susceptibility.

Risk factors for infectious mononucleosis

The factors influencing clinical disease are summarized in Table 53.2.

The marked difference in infectious mononucleosis incidence in comparable settings with an equal number of susceptible individuals, indicates that determinants other than immune status and age at infection are involved. As intimate contact appears to account for most cases of infectious mononucleosis (Crawford *et al.*, 2002), age-specific incidence of infectious mononucleosis in otherwise comparable settings can differ due to behavioural differences.

In children, carriage of the ATA haplotype in the promoter of interleukin (IL)-10, is associated with high levels of spontaneous IL-10 and a late age of primary EBV infection. Thus, the ATA haplotype may increase the age at primary infection and perhaps also the risk of symptomatic disease (Helminen *et al.*, 2001).

Studies on HLA-alleles and infectious mononucleosis have produced conflicting results. A higher frequency of HLA-B-3501 among infectious mononucleosis cases compared to controls has been reported, however, the association between infectious mononucleosis, HLA-B-3501 and other HLA-alleles has not been reproduced in other populations (Chang, 1980).

Viral factors, including EBV strain variation, have not been associated with a different ability to cause infectious mononucleosis.

EBV dynamics during infectious mononucleosis

In healthy seropositive individuals EBV DNA in serum is only occasionally detected, but in the acute phase of infectious mononucleosis high loads of EBV DNA in serum is

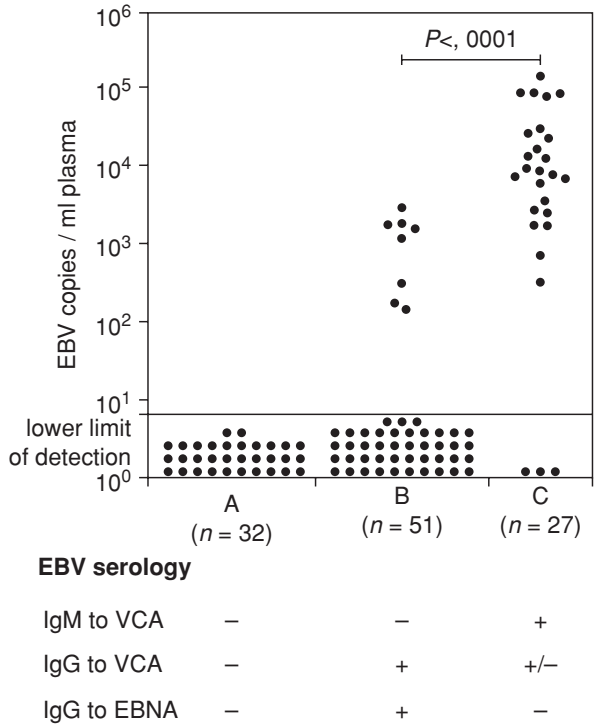

Fig. 53.4. EBV DNA levels in sera from individuals with different EBV-antibody patterns. A: sero-negative, B:sero-positive, C: acute EBV-infection. Reproduced from Berger *et al.*, 2001 with permission from John Wiley & Sons, Inc.

present in most patients (Fig. 53.4) (Berger *et al.*, 2001). The peak in serum viral load is observed in the first seven days of disease, thereafter viral load decreases with resolution of symptoms, although there seem to be considerable inter-individual differences in the decline (Berger *et al.*, 2001). Parallel with the increase in serum viral load, the EBV viral load in peripheral blood mononuclear cells (PBMC) increase to a maximum within the first weeks of the disease, and thereafter declines (Kimura *et al.*, 1999). However, sustained high levels of EBV DNA is found in saliva for at least 6 months after onset of clinical disease, associated with persistent infectivity of saliva (Fafi-Kremer *et al.*, 2005).

Despite the lack of massive expansion in numbers of T lymphocytes in asymptomatic primary EBV infection, both patients with IM and asymptomatic primary infection have similar high EBV viral loads in PBMC. Thus the large T cell expansion seen in IM patients may represent an overreaction, not associated with the control of the primary EBV infection (Silins *et al.*, 2001).

Studies on EBV viral load in primary infection have focused on the time around the infection, and the importance of early versus delayed primary EBV infection on long-term EBV viral load is unknown.

Table 53.3. Proposed guidelines for diagnosing CAEBV (all conditions must be fulfilled). Adapted from Okano *et al.*, 2005 with permission from John Wiley & Sons, Inc.

I.	Persistent or recurrent infectious mononucleosis-like symptoms
II.	Unusual pattern of anti-EBV antibodies with raised anti-VCA and anti- EA, and/or detection of increased EBV genomes in affected tissue, including the peripheral blood
III.	Chronic illness which cannot be explained by other known disease processes at diagnosis*

* EBV-associated diseases such as hemophagocytic lymphohistiocytosis or lymphomas mainly derived from T-cells or NK-cells often develop during the course of illness.

Chronic active EBV infection

Chronic active EBV infection (CAEBV) was first described in the late 1970s, and is characterized by chronic or recurrent infectious mononucleosis-like symptoms and by an unusual pattern of EBV antibodies. Criteria's for diagnosing CAEBV has been suggested earlier, and recently a new set of guidelines has been proposed (Table 53.3) (Okano, *et al.*, 2005; Straus, 1988).

The antibody pattern resembles acute infection and is characterized by high titers of IgG-VCA and IgG-EA, and absence of EBNA antibodies. Patients with CAEBV also have lower frequencies of EBV-specific $CD8^+$ T-cells, compared to infectious mononucleosis patients and healthy individuals (Sugaya *et al.*, 2004). Methods for measuring EBV viral load can be included, as high EBV viral loads in peripheral blood lymphocytes and serum are present (Kimura *et al.*, 2001). There is no known hereditary background, and CAEBV is not associated with mutations in the gene responsible for X-linked lymphoproliferative syndrome (Sumazaki *et al.*, 2001).

CAEBV seem to constitute a disease spectrum with unusual EBV activation, from chronic symptomatic EBV infections with moderately elevated EBV antibodies and a generally good prognosis, to severe chronic active EBV infection with extraordinarily elevated EBV antibodies, clonal expansion of EBV-infected T cells and NK cells, severe clinical and hematological findings, and a generally poor prognosis with high mortality from pancytopenia, lymphoma and hepatic failure (Fig. 53.5) (Kimura *et al.*, 2001; Okano, 2002).

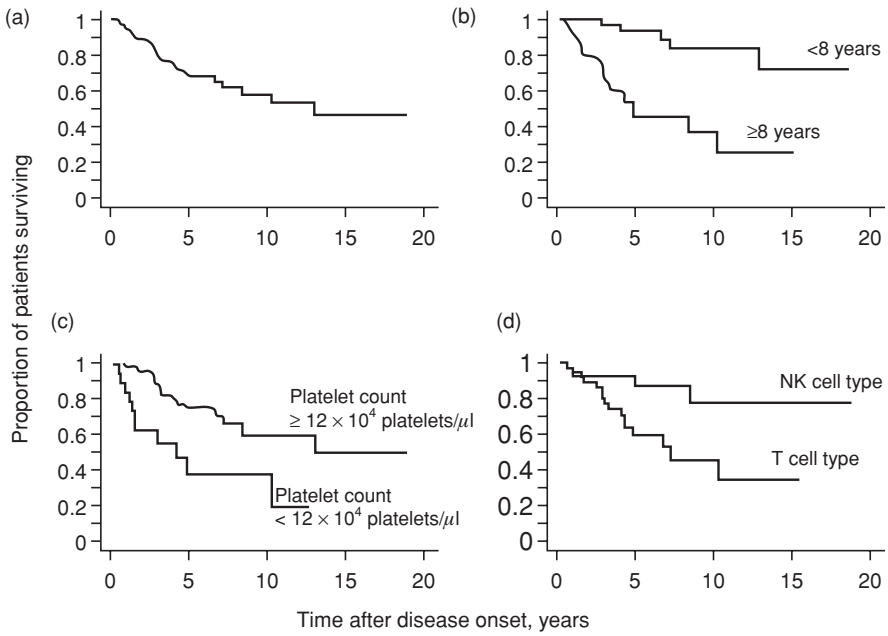

Fig. 53.5. Survival after onset of severe CAEBV. All patients (A), and according to age (B), platelet count (C) and T/NK cell-type of disease (D). Reproduced from (Kimura *et al.*, 2001) with permission from The University of Chicago Press.

Thrombocytopenia and age> = 8 years at onset of disease are associated with a poorer outcome (Kimura *et al.*, 2003).

Epidemiology

Studies on CAEBV have been based on case series, and estimates of population incidences are not available. The disease is very rare, and in 2002 a national survey of severe CAEBV in Japan identified 82 patients (Kimura *et al.*, 2003). Mean age at onset of disease in this survey was 11.3 years with men and women equally represented. Many of the studies on CAEBV have originated in Japan, but whether this reflects a true difference in incidence, or an increased awareness is unknown. However, CAEBV in different geographical areas may be of different entities, as CAEBV in Western populations appears to be milder (Savoldo *et al.*, 2002).

X-linked lymphoproliferative syndrome

X-linked lymphoproliferative syndrome (XLP) or Duncan's disease is a rare, primary immunodeficiency that was first described as a familial disorder affecting males with a rapidly fatal course in response to EBV infection (Purtilo *et al.*, 1975). XLP is characterized by three major phenotypes (Table 53.4): fulminant infectious mononucleosis, B cell lymphoma and dysgammaglobulinemia. Occasionally aplastic anemia, vasculitis and pulmonary lymphomatoid granulomatosis are seen (Engel *et al.*, 2003). A patient can develop more than one phenotype.

Table 53.4. Phenotypes of X-linked lymphoproliferative syndrome. Adapted from Engel *et al.*, 2003 with permission from Nature Publishing Group.

Phenotype	Proportion (%)
Fulminant infectious mononucleosis	50
Following infection with Epstein–Barr virus, patients mount a marked polyclonal expansion of B and T cells leading to destruction of the liver and bone marrow	
B-cell lymphoma	30
Extranodal non-Hodgkin's B-cell lymphomas, usually Burkitt's type, most of which involve the ileocecal region of the intestine	
Dysgammaglobulinemia	30
Acquired dysgammaglobulinemia and other abnormalities in immunglobulin synthesis	

A lack of immune surveillance of EBV in patients with the infectious mononucleosis phenotype of XLP is assumed, however, the phenotypes with B-cell lymphoma and dysgammaglobulinaemia can be observed in patients with or without signs of previous infection with EBV, suggesting

Table 53.5. Effect of EBV infection on clinical phenotype in XLP. Adapted from (Sumegi, *et al.* 2000) with permission from the American Society of Hematology

Phenotype	EBV+ (n = 114)	EBV− (n = 38)	P
Fulminant infectious mononucleosis	70	0	0.0001
Dysgammaglobulinaemia	19	15	0.30
Lymphoproliferative disease	20	21	0.24
Aplastic anemia	5	2	0.03

other antigenic stimuli are also involved in the development of XLP (Table 53.5) (Engel *et al.*, 2003; Sumegi *et al.*, 2000).

Treatment is difficult, haematopoietic stem cell transplantation for fulminant infectious mononucleosis and B-cell lymphomas, and immunoglobulin treatment for agammaglobulinemia has been suggested, but the mortality by the age of 40 years is nearly 100% (Gross *et al.*, 1996; Morra *et al.*, 2001).

The genetic basis for XLP has been identified as an alteration or deletion of the gene SH2D1A, that codes for a cytoplasmatic protein, SAP (SLAM-associated protein, where SLAM is 'signalling lymphocytic activation molecule') (Coffey *et al.*, 1998; Nichols *et al.*, 1998). SAP interacts with several signaling molecules of the SLAM (CD150) family, and is expressed by all T and NK cells. Defective SAP causes selective alterations of the T-/NK-cell function that compromise the ability of these cells to control infection with EBV (Benoit *et al.*, 2000; Engel *et al.*, 2003). Elevated EBV antibody titers are observed in mothers to boys with XLP, although, normal levels of circulating EBV-DNA in XLP patients surviving the initial phase suggest that SAP function is not essential for proper control of EBV replication after primary infection (Sumazaki *et al.*, 2001).

Mutations of SH2D1A are detected in nearly all cases of XLP with a previous family history of XLP, however, mutations of SH2D1A are frequently missing in XLP cases without a family history (Sumegi *et al.*, 2000), although de novo mutations can occur (Sumazaki *et al.*, 2001).

Epidemiology

XLP is estimated to affect approximately 1 in 1,000,000 males (Purtilo *et al.*, 1975). However, this number is likely to be an underestimate, due to the similarity of disease manifestations with other clinically related disorders, such as common variable immunodefiency and the hemophagocytic syndromes (Nichols *et al.*, 2005). The age at onset of clinical disease vary considerably from less than one year to 40 years, with a median of three to eight years (Sumegi *et al.*, 2000). An international XLP registry was established in 1978 and contains over 300 patients from more than 80 families, with up to 17 affected males reported from a single family (Hamilton *et al.*, 1980; Sumegi *et al.*, 2000). XLP patients have been reported from North and South America, Europe and Japan, but it is unknown whether the geographical distribution reflects true differences in incidence or differences in awareness.

Epstein–Barr virus and malignant neoplasms

EBV has been implicated in the development of a wide variety of benign and malignant diseases (Table 53.6). In the following, only the virus' association with malignant diseases will be described. Accordingly, the association between EBV and autoimmune conditions such as multiple sclerosis (Wekerle and Hohlfeld, 2003) or systemic lupus erythematosus (Kang *et al.*, 2004), or immune deficiency-related conditions, such as oral hairy leukoplakia (Niedobitek *et al.*, 1991) and lymphoid interstitial pneumonitis (Swigris *et al.*, 2002) will not be discussed. Focus will be on the main characteristics of EBV-associated cancers and on the evidence linking them with the virus. Nasopharyngeal carcinoma, Burkitt's lymphoma, and Hodgkin's lymphoma will be described in some detail, whereas the association between EBV and other malignant disorders are described more cursorily.

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma is derived from the epithelial lining of the nasopharynx. It typically develops in the lymphoreticular tissue rich area in the fossa of Rosenmüller, and less frequently in the roof and wall of the nasopharynx (IARC, 1997). Histopathologically, two major groups of nasopharyngeal carcinomas are recognized, i.e. keratinizing squamous cell carcinoma (WHO type I), and non-keratinizing carcinoma, the latter being further split up into differentiated (WHO type II) and undifferentiated carcinomas (WHO type III) (Shanmugaratnam, 1991).

Epidemiology

The occurrence of nasopharyngeal carcinoma is characterized by a remarkable geographical and ethnic variation as reflected in the combined occurrence of all types of cancer in the nusopharynx (Table 53.7). (Yu and Yuan, 2002). The

Table 53.6. Evidence for an association between different types of cancer and EBV. Adapted from Hsu and Glaser, 2000

Malignancy	Lines of evidence
Nasopharyngeal carcinoma	• Elevated anti-EBV antibody titers preceding and at diagnosis • Elevated levels of free EBV DNA at diagnosis • Correlation between anti-EBV antibodies and free virus DNA in plasma, and tumor burden and prognosis • Demonstration of monoclonal EBV in tumor cells
Lymphomas Burkitt's lymphoma variants	• Elevated anti-EBV antibody titers preceding and at diagnosis • Demonstration of monoclonal EBV in tumor cells • Increased occurrence in immune suppressed patients
AIDS associated lymphoma [other than Burkitt' lymphoma	• Increased occurrence compared with general population • Overall risk correlates with immune function • Demonstration of monoclonal EBV in malignant cells
Post-transplant lymphoma	• Increased occurrence compared with general population • Overall risk correlates with immune function • Demonstration of monoclonal EBV in malignant cells
Nasal T/NK lymphoma	• Demonstration of monoclonal EBV in tumor cells
Hodgkin's lymphoma	• Increased risk in infectious mononucleosis patients • Increased risk in immune suppression • Elevated anti-EBV antibody titres preceding and at diagnosis • Demonstration of mononclonal EBV in malignant cells
Gastric adenocarcinoma	• Elevated anti-EBV antibody titers preceding and at diagnosis • Demonstration of monoclonal EBV in tumor cells
Lymphoepithelioma-like carcinoma	• Demonstration of monoclonal EBV in malignant cells
Leiomyosarcoma	• Increased risk with immunosuppression • Demonstration of mononclonal EBV in malignant cells

tumor is quite rare in most Western countries with incidence rates less than 1 per 100 000 persons per year, as is it indeed in most parts of the world, but high incidence rates are observed in certain ethnic populations in South-East Asia and North Africa and in the circumpolar indigenous populations (Table 53.7). Common to both low-and high incidence areas, nasopharyngeal carcinoma is seen two-to-three times more often in men than in women (Table 53.7). Different age-specific incidence patterns are observed in endemic and non-endemic regions (Parkin *et al.*, 2002; Lee *et al.*, 2003; Yu and Yuan, 2002): In non-endemic regions nasopharyngeal carcinoma occurrence increases continuously with age, whereas in endemic regions the incidence increases with age to peak around the age of 50 years and decrease thereafter. A third bimodal age pattern with a minor incidence peak in adolescents and young adults has been described in some populations with low to intermediate nasopharyngeal carcinoma incidence (Yu and Yuan, 2002; Daoud *et al.*, 2003).

Even within regions where nasopharyngeal carcinoma is endemic considerable variation in disease occurrence can be observed between different ethnic subpopulations. For instance, in the Chinese province of Guangdong the incidence of nasopharyngeal carcinoma is twice as high in Cantonese as in other ethnic groups (Li *et al.*, 1985; Yu *et al.*, 1981; Yu and Yuan, 2002). Familial clustering of nasopharyngeal carcinoma and other cancers is well-established from a plethora of case-reports (Zeng and Jia, 2002). The familial accumulation in turn translates into increased risks for nasopharyngeal carcinomas, e.g., first degree-relatives of Greenlandic Inuits with nasopharyngeal carcinoma have an eight-fold higher risk of the tumor than the general population (Friborg *et al.*, 2005). Epidemiologically, these observations may indicate a genetic predisposition to nasopharyngeal carcinoma, a suspicion that has been supported by genetic studies. Specifically, a meta-analysis of published data for Chinese patients showed increased risk for HLA alleles A2, B14, B46, and decreased

Table 53.7. Incidence rates for nasopharyngeal cancer (all types combined) in different regions. Adapted from Yu and Yuan, 2002 and updated from Parkin, *et al.*, 2002. Data for Greenland from Friborg *et al.*, 2003

	Age-standardized (world) incidence rates (per 100,000)		
Population	Males	Females	Calendar period
Chinese, Hong Kong	21.4	8.3	1993 to 1997
Chinese, Tapei	8.9	3.4	1997
Chinese, Shanghai	4.2	1.5	1993 to 1997
Chinese, Tianjin	1.7	0.5	1993 to 1997
Inuits, Greenland	10.3	8.0	1988 to 1997
Inuits, Athabascans, and Aleuts, Alaska	11.9	5.6	1980 to 1987
Thais, Chiang Mai	3.2	1.3	1993 to 1997
Vietnamese, Hanoi	10.4	4.6	1993 to 1997
Malays, Singapore	6.8	2.0	1993 to 1997
Filipinos, Manila	7.2	2.5	1993 to 1997
Kuwaits, Kuwait	2.6	0.9	1994 to 1997
Algerians, Setif	8.0	2.7	1990 to 1993
Israeli Jews born in Morocco, Algeria or Tunisia	2.8	1.3	1961 to 1981
U.S. Whites (SEER)	0.5	0.2	1993 to 1997

risks for HLA alleles A11, B13 and B22 (Goldsmith *et al.*, 2002). Moreover, susceptibility *loci* have been reported on chromosomes 3 (Xiong *et al.*, 2004), 4 (Feng *et al.*, 2002), 14 [Diehl *et al.*, unpublished observations quoted in (Pickard *et al.*, 2004)] and near the HLA-locus (Lu *et al.*, 1990). Polymorphisms in genes coding for certain enzymes involved in nitrosamine metabolism [Gluthathione S-transferase M1 and cytochrome P450 2E1] also have been reported to correlate with risk for nasopharyngeal carcinoma [reviewed by (Zeng and Jia, 2002; Hildesheim *et al.*, 1997)].

There is good evidence, however, that environmental factors are also significant to the risk of nasopharyngeal carcinoma. Accordingly studies of families emigrating from high to low risk regions have shown that the risk of nasopharyngeal carcinomas decreases between successive generations (IARC, 1997). Among environmental factors, the evidence is particularly strong against certain diets including Cantonese-style salted fish and other preserved foods (for review, see IARC, 1997). Accordingly, a high intake of preserved foods is a common characteristic of the populations where nasopharyngeal carcinoma is endemic, and case-control studies in endemic as well as non-endemic regions have demonstrated an association between intake of such food items and nasopharyngeal carcinoma risk. Moreover, the risk for nasopharyngeal carcinomas seems to be inversely correlated with the age of first exposure. Consistent with the role of diet, the incidence of nasopharyngeal carcinoma in Hong Kong has decreased over the last decades concomitantly with changes in lifestyle towards a western-world pattern (Lee *et al.*, 2003). Other suggested risk factors have included low socioeconomic status (possibly correlated with high intake of preserved food items), tobacco, and alcohol, and occupational exposure to formaldehyde and wood dust (Hildesheim *et al.*, 2001; IARC, 1997).

It is noteworthy that the association between preserved food and risk for nasopharyngeal carcinoma gains biological plausibility in the context of the familial accumulation of the tumor and its association with EBV. Accordingly, the preserved foods contain carcinogenic nitrosamines, as well as EBV-activating substances (IARC, 1997).

Evidence of association with EBV

Infection with EBV has been implicated in the development of nasopharyngeal carcinoma by several different lines of evidence (Table 53.6). Historically, the first indication was the observation that sera from African and American patients with nasopharyngeal carcihoma were often more positive for precipitating antibodies to antigens prepared from cultured Burkitt's lymphoma cells than controls (Old *et al.*, 1966). This observation has since been confirmed in serological studies showing elevated titers of IgG and in particular IgA antibodies against EBV viral capsid, early and nuclear antigens in nasopharyngeal carcinoma patients data being less compelling for type I than types II and III, manifesting as apparent ethnic variations (IARC, 1997; Raab-Traub, 2000). Antibody titers correlate with stage of disease and has been shown to return to normal levels in long-term disease-free survivors (Yu and Henderson, 2004). More compelling than the sero-prevalence surveys in patients are, however, the results of a prospective study of 9699 persons which showed that presence of IgA anti-EBV viral capsid antigen antibodies or neutralising EBV specific anti-DNase antibodies correlated with subsequent risk for nasopharyngeal carcinoma (Fig. 53.6) (Chien *et al.*, 2001).

The serological findings are corroborated by the demonstration of monoclonal EBV in the malignant nasopharyngeal carcinoma cells (Raab-Traub and Flynn, 1986). This line of evidence is most consistent for nasopharyngeal carcinoma types II and III, but the virus has also been demonstrated in type I carcinomas (Nicholls *et al.*, 1997; Raab-Traub, 2000). Consistent with the assumption of a causal role for the virus in development of the tumor, monoclonal

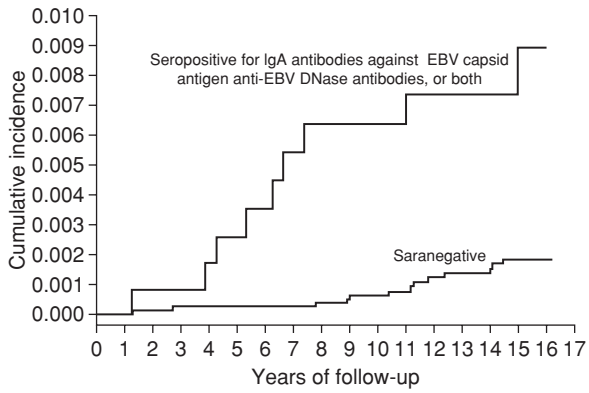

Fig. 53.6. Cumulative incidence of nasopharyngeal carcinoma in persons testing positive or negative for either IgA anti-Epstein–Barr virus viral capsid or neutralizing anti-Epstein–Barr virus DNase antibodies. Reproduced from Chien *et al.*, 2001 with permission from the Massachusetts Medical Society.

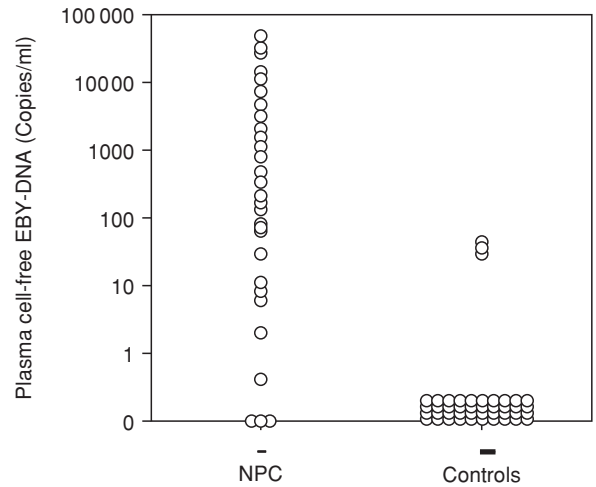

Fig. 53.7. Comparison of plasma cell-free EBV DNA in NPC patients and control subjects. The categories (NPC patients and control subjects) are plotted on the X-axis. The Y-axis denotes the concentration of cell-free EBV DNA (copies of EBV DNA/ml of plasma) detected by the BamHI-W region PCR system). Reproduced from (Lo *et al.*, 1999) with permission from the American Association for Cancer Research.

EBV has also been demonstrated in pre-invasive dysplastic and carcinoma *in situ* lesions (Pathmanathan *et al.*, 1995) as has the virus been demonstrated in nasopharyngeal carcinoma metastases (Lee *et al.*, 2000).

More recently, the detection and quantification of circulating EBV DNA have attracted interest. In one study, such DNA was demonstrated in 96% of patients with nasopharyngeal carcinoma as compared with only in 7% of controls, and levels of DNA moreover correlated with disease stage (Figure 53.7) (Lo *et al.*, 1999) and, independently hereof, also with prognosis (Lo *et al.*, 2000; Lin *et al.*, 2004).

Tumors of the lymphoid tissues

The tumors of the lymphoid tissues constitute a clinically and epidemiologically heterogeneous group of malignancies with the common characteristic that they are derived from cells belonging to the lymphoid lineage. Three major categories of lymphoid malignancies are recognized today, i.e. B-cell lymphomas, T and NK-cell lymphomas, and Hodgkin's lymphomas (Harris *et al.*, 2001a,b). Within each of these categories, distinct disease entities are recognised based on morphologic, immunophenotypic, genetic and clinical characteristics.

The occurrence of non-Hodgkin's lymphoma generally increases with age and overall lymphomas are more often seen in men than in women with a male : female incidence ratio of 1.5–2:1 (Parkin *et al.*, 2002). Incidence rates vary internationally, age-standardized (world) rates ranging from typically 3–8 and 2–6 per 100 000 Asian men and women to 10–15 and 5–10 per 100 000 in European men and women (IARC, 2002). An as yet unexplained remarkable increase in the incidence of all types of non-Hodgkin' lymphomas combined was apparent in the latter half of the twentieth century in most regions of the world, amounting to 3%–4% increase annually (Devesa and Fears, 1992). Recent data from Scandinavia suggest that the increase has just as inexplicably begun to subside (Sandin *et al.*, 2006).

Relatively few risk factors have been established for non-Hodgkin's lymphomas (Scherr and Mueller, 1996; Grulich and Vajdic, 2005; Ekstrom-Smedby, 2006). In part, this may reflects that the composite nature of the malignant lymphomas including possible etiological heterogeneity has not always been taken into consideration previously. The most consistently observed risk factor is immune suppression, primary as well as acquired. Other risk factors include familial aggregation (Chang *et al.*, 2005; Goldin *et al.*, 2005), autoimmune conditions (Zintzaras *et al.*, 2005) and exposure to certain hair dyes (Takkouche *et al.*, 2005) and herbicides (Fritschi *et al.*, 2005; Scherr and Mueller, 1996). Several infectious organisms are known or suspected to be etiologically linked to lymphoma development including both bacteria, e.g., *Helicobacter pylori* (Wotherspoon *et al.*, 1991), *Borrelia burgdorferi* (Cerroni *et al.*, 1997), *Campylobacter jejuni* (Lecuit *et al.*, 2004), *Chlamydia psittaci* (Ferreri *et al.*, 2004) and viruses, e.g., human herpesvirus-8 (Cesarman *et al.*, 1995), human T-cell lymphotropic virus type I (Hinuma

et al., 1981), hepatis C virus (Pozzato *et al.*, 1994), and EBV (Epstein *et al.*, 1964).

The evidence for an association with EBV infection is the strongest for Burkitt's lymphomas, NK/T-cell lymphomas of the nasal cavity, for malignant lymphomas in immune incompetent patients, and for a subset of Hodgkin's lymphomas. The virus may, however, also be encountered in other types of malignant lymphomas, though less regularly (IARC, 1997). It is noteworthy, therefore, that in a prospective serological investigation elevated IgG ($\geq$ 1:320) and IgM ($\geq$ 1:5) titres of anti-viral capsid antigen antibodies were associated with 2.5-(IgG)and 3.2-fold (IgM) increased risks for non-Hodgkin's lymphoma overall with no apparent difference between different lymphoma subtypes (Mueller *et al.*, 1991).

Burkitt's lymphoma/leukemia

Burkitt's lymphoma is a highly aggressive lymphoma that often presents extranodally or as acute leukemia (Diebold *et al.*, 2001). The presumed cell of origin is a germinal centre B-cell. Based on clinical and epidemiological characteristics, three variants of Burkitt's lymphoma are recognized, i.e., endemic, sporadic, and immunodeficiency-associated Burkitt's lymphoma. Histologically, the tumor is generally characterized by monomorphic cytoarchitecture composed of medium-sized B cells with basophilic cytoplasm and numerous mitotic figures with variations between the tumor variants. A constant feature shared by all Burkitt's lymphoma variants is chromosomal translocations involving the MYC oncogene on chromosome 8 (i.e., either t(8:14), t(2:8) or t(8:22) (Diebold *et al.*, 2001)).

Epidemiology of endemic Burkitt's lymphoma

The endemic variant of Burkitt's lymphoma is primarily a childhood malignancy seen in Papua, New Guinea and in equatorial Africa, where in certain areas it is the most common childhood cancer (van den Bosch, 2004). The tumor occurs two-to-three times as often in boys as in girls and in both genders the incidence of endemic Burkitt's lymphoma peaks at ages 5–9 years (Diebold *et al.*, 2001). Precise incidence rates are difficult to obtain, but data suggest crude incidence rates of 4.6 and 2.9 per 100 000 in Ugandan boys and girls < 15 years (IARC, 2002).

One of the most striking characteristics of endemic Burkitt's lymphoma is the correspondence between its geographical distribution and measures of prevalence of malaria infection, a correlation that is apparent both between and within regions and over time (IARC, 1997). Moreover, the peak ages for endemic Burkitt's lymphoma is also the age interval during which anti-malarial antibodies peak (IARC, 1997). These ecological similarities have been interpreted as reflecting a role for malarial infection in development of endemic Burkitt's lymphoma, as discussed below. Other suspected risk factors, the effects of which are also related to EBV infection, are certain groups of plants used in traditional medicine that may stimulate viral replication. The direct evidence of the role of such is, however, limited (IARC, 1997).

Epidemiology of sporadic Burkitt's lymphoma

Sporadic Burkitt's lymphoma occurs predominantly in children and young adults, and is seen throughout the world (Diebold *et al.*, 2001). The tumor make up a few percent of all lymphomas in industrialized countries, but constitutes up to 50% of all lymphomas in children (Diebold *et al.*, 2001). Like the endemic variant, sporadic Burkitt's lymphoma is seen two-to-three times as often in males as in females. Generally, the incidence of sporadic Burkitt's lymphoma is much lower than that of the endemic variant, e.g., rates of 0.38 and 0.08 per 100 000 are observed in white US boys and girls <15 years (Parkin, 2002). Familial accumulation of the lymphoma has been reported in a few instances, but otherwise few risk factors have been established (IARC, 1997).

Epidemiology of AIDS-related Burkitt's lymphoma

Burkitt's lymphoma frequently is the AIDS defining malignancy in HIV-infected patients (Diebold *et al.*, 2001). The tumor and its association with EBV are described later. Burkitt's lymphoma is also seen in organ transplant recipients.

Evidence of association with EBV

EBV was originally identified in an endemic Burkitt's lymphoma cell culture (Epstein *et al.*, 1964), and of the three lymphoma variants, the endemic type has remained the most strongly associated with the virus. The evidence of an association between Burkitt's lymphoma and EBV includes both serological and molecular biological studies (Table 53.6).

An early classical paper describes a prospective serological investigation set in the Uganda West Nile District, where Burkitt's lymphoma is endemic. Based on an initial collection of blood samples from nearly 42 000 healthy children, children who subsequently developed Burkitt's lymphoma displayed statistically significantly higher titres of antiviral capsid antigen antibodies than their peers, who remained healthy (geometric mean titre 425.5 vs. 125.8). No differences were observed between the two groups of children for anti-early antigen or anti-EBV nuclear antigen antibodies (de-Thé *et al.*, 1978). In a later update of the study, each twofold dilution of antiviral capsid antigen antibody titer

was associated with a five-fold in Burkitt's lymphoma risk overall and a nine-fold increased risk of EBV-positive lymphomas (Geser *et al.*, 1982).

Employing different serological techniques, studies have suggested that African Burkitt's lymphoma patients are more often infected with EBV than their peers and also display higher anti-EBV antibody titres (IARC, 1997). Similar patterns have also been observed in sporadic Burkitt's lymphoma patients, in particular children, though with less striking differences *vis à vis* controls (IARC, 1997).

Large case-series have since the original observation confirmed the presence of EBV in endemic Burkitt's lymphoma cells. Accordingly, virus has been demonstrated in more than 95% of investigated endemic Burkitt's lymphoma cases (IARC, 1997). In contrast, the prevalence of EBV in sporadic Burkitt's lymphoma in general appears to be less than 30% (IARC, 1997; Diebold *et al.*, 2001), albeit with considerable variation in reported estimates ranging between 15 and 88% (Hsu and Glaser, 2000). In AIDS-related Burkitt's lymphoma, the prevalence of EBV is 25 to 50% (Diebold *et al.*, 2001; Raphael *et al.*, 2001).

The mechanism by which EBV contributes to the development of Burkitt's lymphoma is not entirely understood, but the virus's absence in many cases either suggests that it is neither sufficient nor necessary for the tumor to develop or, alternatively, that EBV-positive and -negative tumors are different biological entities (Bellan *et al.*, 2005). For EBV positive tumors, age at infection with the virus and the ability to control infected cells seem critical (Mueller *et al.*, 1996). For endemic Burkitt's lymphoma it has been suggested that early EBV infection may lead to transformation and replication of a large subset of B-lymphocytes. This process in turn may be augmented by recurrent malaria infections that act both as B-cell mitogen and T-cell suppressant. Translocation involving chromosome 8 may then result from the increased cell replication (Klein, 1979; de The, 2000). Some support for the interaction between malaria and EBV comes from studies showing that the number of EBV infected cells is higher during acute malaria than after recovery (Lam *et al.*, 1991) and that the number of EBV infected cells correlate with the intensity of malaria transmission in the area of residence (Moormann *et al.*, 2005). It is noteworthy, however, that in the aforementioned prospective serological investigation, the children who developed endemic Burkitt's lymphoma and children who remained healthy had similar rates of malaria parasitemia before tumor development (de-Thé *et al.*, 1978). Accordingly, other models for the role of EBV in Burkitt's lymphoma development have also been proposed (Hecht and Aster, 2000; van den Bosch, 2004). The occurrence of EBV-positive Burkitt's lymphoma in AIDS patients and in organ transplant recipients would also be consistent with a critical role for immunological control of EBV-infected cells in the lymphoma development, as would an inverse correlation between socioeconomic status and Burkitt's lymphoma occurrence (Hsu and Glaser, 2000).

NK/T-cell lymphomas

EBV is associated with certain types of NK/T-cell lymphomas. These include in particular the extranodal NK/T-cell lymphoma of the nasal type, but also aggressive NK-cell leukemia (Nava and Jaffe, 2005) and possibly angioimmunoblastic T-cell lymphoma (Anagnostopoulos *et al.*, 1992; Chan *et al.*, 1999; Huh *et al.*, 1999; Weiss *et al.*, 1992) and extranodal enteropathy-type T-cell lymphoma (Huh *et al.*, 1999; Quintanilla-Martinez *et al.*, 1997; Zhang *et al.*, 2005). Histologically, extranodal NK/T-cell lymphomas of the nasal type are characterized by a broad morphological spectrum, but an angiodestructive pattern with frequent necrosis and apoptosis is a characteristic finding (Chan *et al.*, 2001; Nava and Jaffe, 2005). As signaled by the name the malignant cells are either of NK-cell (the majority) or T-cell origin. The nasal region is the most frequent site of involvement, but the tumor may also present at other extranodal sites such as skin testis, kidney, upper gastrointestinal tract, and the orbit (Chan *et al.*, 2001; Rizvi *et al.*, 2006).

Epidemiology of mature T- and NK-cell lymphomas

Mature T- and NK-cell tumors are generally rare tumors, and NK/T cell lymphomas of the nasal type even more so. In an international series comprising lymphoma patients from the US, Europe, Asia and South Africa peripheral T-cell lymphoma made up 9.4% of all non-Hodgkin's lymphomas, however, with considerable geographic variation, ranging from 1.5% in Vancouver, Canada to 18.3% in Hong Kong (Rudiger *et al.*, 2002). In the same series, NK/T-cell lymphomas of the nasal type constituted a mere 1.4% of the all investigated lymphomas (Rudiger *et al.*, 2002), all cases except three diagnosed in Hong Kong. Thus, NK/T-cell lymphomas of the nasal type are rare in the Western world, and is more commonly seen in Asia, Mexico and in Central and South America countries (Hsu and Glaser, 2000). In a recent Chinese case series from Hong Kong, NK/T-cell lymphomas of the nasal type made up 6.3% of all non-Hodgkin's lymphomas (Au *et al.*, 2005), and similar proportions have been reported from Peru (Quintanilla-Martinez *et al.*, 1999). Generally, the incidence is held to be higher in men than in women (Hsu and Glaser, 2000). Besides the ethnic variation little is known about risk factors for this small

group of lymphomas, but the entity has been described in immune dysfunctional individuals (Stadlmann, 2001).

Evidence of association with EBV

EBV has been incriminated in NK/T-cell lymphoma development exclusively by the demonstration of the virus in the tumor cells (Table 53.6). Accordingly, patient series have shown that the NK/T-cell lymphomas of the nasal type almost invariably (90%) harbor EBV, irrespective of the patient's ethnicity (Chan *et al.*, 2001a,b; Kanavaros *et al.*, 1993; Miyazato *et al.*, 2004; Quintanilla-Martinez *et al.*, 1997); in Asians also when presenting outside the nasal cavity (Chan *et al.*, 1997).

Hodgkin's lymphoma

Hodgkin's lymphoma develops from germinal center B-lymphocytes in the vast majority (> 98%) of all cases, and in rare instances from post-thymic T-cells (Stein *et al.*, 2001). Histologically, the tumor typically contains a small number of malignant cells, which are large mono- and multinucleated cells (Hodgkin's or Reed–Sternberg cells), surrounded by T-lymphocytes in a rosette-like pattern and dispersed in an abundant mixture of reactive inflammatory and accessory cells (Stein, 2001). Based on clinical and biological criteria, two main types of Hodgkin's lymphoma are recognized, i.e., nodular lymphocyte predominant (5%) and classical (95%) Hodgkin's lymphoma, the latter being further divided into four histological subtypes [nodular sclerosing (70%), mixed cellular (20–25%), lymphocyte rich (∼ 5%) and lymphocyte depleted (<5%) classical Hodgkin's lymphoma (Stein, 2001).

Epidemiology

Hodgkin's lymphomas constitute 10%–15% of all malignant lymphomas [slighty more when chronic lymphocytic leukemia is disregarded] (Parkin *et al.*, 2002). In Western countries, age-standardized (world) incidence rates are typically in the range of 2–4 per 100 000 in men and 1.5–3 per 100 000 in women, whereas incidence rates < 1 per 100 000 in both men and women are typical for Asia (Parkin *et al.*, 2002). Geographical differences also exist with respect to age-specific incidence rates. In industrialized countries a conspicuous bimodal age distribution with cases accumulating in young adults and the elderly has become one of the lymphomas distinguishing characteristics (Fig. 53.8). In Hodgkin's lymphoma epidemiology literature, this pattern has been referred to as Pattern III, implying the existence of Patterns I and II (Correa and O'Conor, 1971). Of these, Pattern I was seen in developing countries and was characterized by relatively high incidence of Hodgkin's lymphoma in children, low incidence in the third decade and high incidence in the elderly. Pattern II was perceived as an intermediate between Pattern I and III. A Pattern IV describing a general paucity of Hodgkin's lymphoma in all age groups was also suggested based on data originally reported from Asian countries (Correa and O'Conor, 1971). A more recent survey of register data indicate that, with the possible exception of Asian countries, these archetypical incidence patterns may no longer be as clearly distributed geographically (Macfarlane *et al.*, 1995).

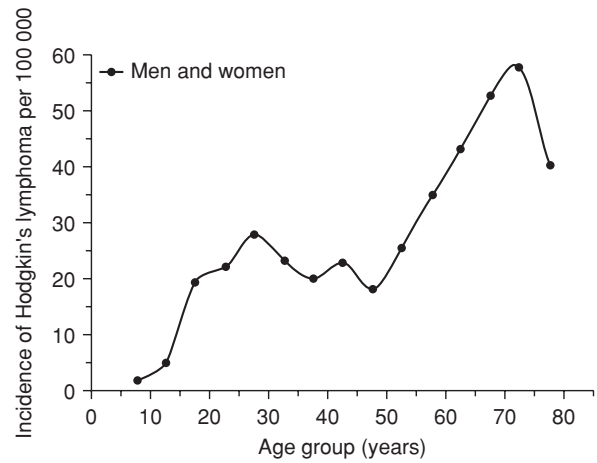

Fig. 53.8. Age-specific incidence of Hodgkin's lymphoma in the white population of Brooklyn, U.S., 1943–52. Reproduced from MacMahon, 1957 with permission from John Wiley & Sons, Inc.

The bi-modal age distribution in Pattern III reflects the age-related distributions of the different subtypes of Hodgkin's lymphoma with the nodular sclerosis subtype making up the bulk of the young adult age peak, and mixed cellularity subtype increasing in frequency with age, but has nevertheless also been used to define epidemiologically (meaningful) disease entities (MacMahon, 1957; MacMahon, 1966). Accordingly, studies of risk factors for Hodgkin's lymphoma have often focused on specific age-groups, i.e., children [<15 years], young adults [15–ca. 44 years], and elderly [≥ca. 45 years], rather than specific Hodgkin's lymphoma subtypes. Presumably reflecting the age distribution of cases, most epidemiological studies have concentrated on Hodgkin's lymphoma in young adults, the risk of which has been associated with an affluent childhood social environment, as measured by long maternal education, small sibling size and housing (Mueller, 1996). In contrast, Hodgkin's lymphoma in children appears to be associated with low socioeconomic status, whereas it plays little if any role for the risk for Hodgkin's lymphoma in the elderly (Mueller, 1996). It has long been suspected that the

association with childhood environment in young adults cases reflects a surrogate for loads of infectious diseases in childhood, and that, in young adults, the lymphoma might arise as an untoward reaction to delayed exposure to a common childhood infectious agent (Gutensohn and Cole, 1977). Consistent with this notion, attendance of nursery school or day care for more than 1 year was associated with a reduced risk (odds ratio = 0.64; 95% confidence interval 0.45 to 0.92) for Hodgkin's lymphoma at ages 15–54 years in a recent investigation (Chang *et al.*, 2004). Patients suffering from immune incompetence, whether acquired or inherited, are at increased risk for Hodgkin's lymphoma. In AIDS patients, for instance, the increase in the order of 10-fold (Frisch *et al.*, 2001). Hodgkin's lymphoma is also known to cluster within families suggesting a genetic predisposition to the disease (Goldin *et al.*, 2004). Also, smoking has recently been incriminated in two large case-control studies, suggesting relative risks of around two for current smokers (Briggs *et al.*, 2002; Chang *et al.*, 2004), although previous studies have yielded conflicting results (for review see Briggs *et al.*, 2002). Among investigated occupational exposures, wood working and formaldehyde exposure have frequently been associated with risk for Hodgkin's lymphoma (Mueller, 1996).

Evidence of association with EBV

Epidemiological, serological, and molecular biological studies have all suggested that EBV is involved in the development of at least a proportion of Hodgkin's lymphomas (Table 53.6). Consistent with suspected mechanisms underlying the association with affluence in childhood, history of infectious mononucleosis has been associated with an increased risk for Hodgkin's lymphoma in cohort as well as case-control studies, relative risks typically in the order of two-to-threefold increased (Alexander *et al.*, 2003; Hjalgrim *et al.*, 2000; IARC. 1997). The risk increase seems to be specific to Hodgkin's lymphoma and inversely correlated with time since infectious mononucleosis, in practice restricting it to the young adult age group (Hjalgrim *et al.*, 2000). Serological investigations have also pointed to a role for EBV in Hodgkin's lymphoma pathogenesis. Specifically, though patients with Hodgkin's lymphoma appear not to be more frequently infected with the viruses (as measured by prevalence of antiviral capsid antigen IgG antibodies at diagnosis) than comparable controls, the patients have higher mean titres of these antibodies (IARC, 1997). In case-control studies the patients also demonstrate antibodies against the early antigen complex and at higher titres more often than normal persons (IARC, 1997). Perhaps the most compelling evidence, however, comes from a prospective serological investigation, in

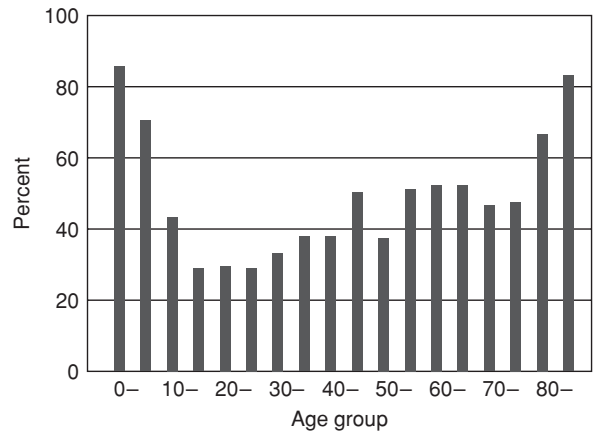

Fig. 53.9. Percentage of Hodgkin's lymphomas positive for EBV by five-year age group (Reproduced from (Glaser *et al.*, 1997)) with permission from John Wiley & Sons, Inc.

which prediagnostic elevated titres of antiviral capsid antigen IgG antibodies (relative risk = 2.6; 90% CI 1.1 to 6.1), anti-diffuse early antigen antibodies (relative risk = 2.6; 90% CI 1.1 to 6.1) and anti-restricted early antigen (1.9; 90% CI 0.90–4.0) were all associated with risk of Hodgkin's lymphoma (adjusted for IgM). In multivariate analyses including all types of anti-EBV antibodies, risk for Hodgkin's lymphoma was associated with high titers of anti-EBV nuclear antigen antibodies (relative risk = 6.7; 90% CI 1.8 to 25) and inversely associated with IgM antibodies (relative risk = 0.07; 90% CI 0.01 to 0.53) (Mueller *et al.*, 1989). The third line of evidence of an association between EBV and Hodgkin's lymphoma is the demonstration of the virus in the malignant cells (Weiss *et al.*, 1987). Importantly, however, the virus is not invariably present in the malignant cells, and the proportion of EBV-positive tumors varies by histological subtype (more common in mixed cellularity than nodular sclerosis Hodgkin's lymphoma), age (less common in young adults than other age groups), sex (more common in men than in women), and geography (more common in developing than developed countries) (Figures 53.9 and 53.10) (Cartwright and Watkins, 2004; Glaser *et al.*, 1997).

The role of EBV in Hodgkin's lymphoma pathogenesis remains uncertain from epidemiological evidence, but there is some evidence to suggest that risk factors may differ between virus-positive and -negative tumors. Accordingly, in some investigations the increased risk for Hodgkin's lymphoma following infectious mononucleosis may be restricted to virus-positive subtypes (Alexander *et al.*, 2000; Hjalgrim *et al.*, 2003), although other investigations have reported no particular predilection for

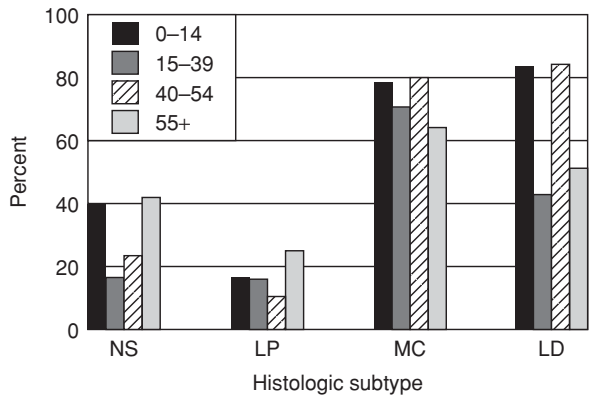

Fig. 53.10. Distribution of EBV positive tumors by histological subtype (Reproduced from (Glaser *et al.*, 1997)) with permission from John Wiley & Sons, Inc.

virus-positive tumors after infectious mononucleosis (Alexander *et al.*, 2003; Sleckman *et al.*, 1998).

It has been speculated that the Hodgkin's lymphoma-mononucleosis association may merely reflect the significance of high socioeconomic status. This speculation is, however, not easily compatible with observations showing that first-degree relatives of mononucleosis patients either have no increased Hodgkin's lymphoma risk (Hjalgrim *et al.*, 2002), or an increased risk for only for virus-positive tumors (Alexander *et al.*, 2003). Also, titres of antiviral capsid antigen antibodies have recently been found to correlate with Hodgkin's lymphoma virus status in both case-control (Alexander *et al.*, 2003; Chang *et al.*, 2004a,b) and prospective studies (Levin *et al.*, 2002). There is also evidence to suggest that smoking is particularly associated with an increased risk for EBV-positive Hodgkin's lymphoma (Briggs *et al.*, 2002; Chang *et al.*, 2004a,b; Glaser *et al.*, 2004), as is low socioeconomic status and race for Hodgkin's lymphoma in childhood (Flavell *et al.*, 2001) and immune-incompetence (Audouin *et al.*, 1992). Finally, inherited susceptibility to EBV-positive Hodgkin's lymphoma has also recently been proposed (Diepstra *et al.*, 2005). None of these different lines of evidence can, however, rule out the simple explanation that the proportion of virus-positive lymphomas merely reflect the number of circulating EBV-infected cells at initiation of lymphoma development (Thorley-Lawson and Gross, 2004).

Lymphoproliferative disease associated with immunodeficiency

The recent WHO classification for tumors of the hematopoietic and lymphatic tissues recognizes four broad clinical settings of immune deficiency that are associated with an increased risk for malignant lymphomas and other lympoproliferative disorders. These are (1) primary immune disorders and deficiencies (Borisch *et al.*, 2001), (2) infection with human immunodeficiency virus (HIV) (Raphael *et al.*, 2001), (3) iatrogenic immune suppression in organ or bone marrow transplant recipients (Harris *et al.*, 2001a,b), and (4) iatrogenic immune suppression associated with methotrexate treatment (Harris and Swerdlow, 2001), typically for auto-immune conditions. With the exception of post-transplant lymphoproliferative disorder, the malignancies observed in these settings are largely similar to sporadic occurring neoplasms.

Primary immune disorders

Primary immune disorders are associated with a substantial risk for malignant disease, reported absolute risks ranging from 12%–25% in patients with Wiskott-Aldrich syndrome, ataxia telangiectasia, and common variable immunodeficiency (Filipovich *et al.*, 1992). Hematopoietic malignancies make up nearly 70% of the observed tumors, clearly different from the normal 8% (Mueller, 1999). Onset of disease is typically in childhood (Borisch *et al.*, 2001). EBV infection plays a significant role in many, though not all, of the malignant lymphomas occurring in patients with primary immune deficiencies (Filipovich *et al.*, 1992) Specifically, loss of immunological control of EBV-infected lymphocytes allows their continued proliferation and malignant transformation, possibly promoted by defective immune regulation and chronic immune stimulation (Filipovich *et al.*, 1992).

Post-transplant lymphoproliferative disease

"Post-transplant lymphoproliferative disorder" (PTLD) in reality defines a spectrum of lymphoid hyperproliferative states that may be observed in solid organ and bone marrow transplant recipients (Loren *et al.*, 2003). PTLDs are most often but not invariably of B-lymphocyte origin, and manifest heterogeneously, possibly reflecting serial development of the disorder (Harris *et al.*, 2001a,b) (Table 53.9). Histologically, it has been suggested that EBV-associated PTLD should include two of the following three features: disruption of underlying architecture by a lymphoproliferative process, presence of monoclonal or polyclonal cell populations, and evidence (typically by *in situ* hybridization for virus-encoded RNA) of EBV in many of the cells (Paya *et al.*, 1999). Although it also includes reactive hyperplasia, the term PTLD is normally used in reference to the malignant end of the PTLD spectrum unless otherwise specified (Green, 2001; Loren *et al.*, 2003).

Table 53.8. Distribution of tumors by primary immunodeficiency syndrome in a combined series of patients with inherited immune deficiencies (Reproduced from Filipovich *et al.*, 1992; Mueller, 1999.) The table is not exhaustive with respect to immune deficiencies carrying increased cancer risk

Immunodeficiency	Total tumors	NHL	Hodgkin's disease	Leukemia	Other tumors
Severe combined immunodeficiency	42	31 (73.8)[a]	4 (9.5)	5 (11.9)	2 (4.8)
Hypogammaglobulinemia	21	7 (33.3)	3 (14.3)	7 (33.3)	4 (19.0)
Common variable immunodeficiency	120	55 (45.8)	8 (6.7)	8 (6.7)	49 (40.8)
IgA deficiency	38	6 (15.8)	3 (7.9)	0 (0)	29 (76.3)
Hyper-UgM syndrome	16	9 (56.3)	4 (25.0)	0 (0)	3 (18.8)
Wiskott-Aldrich syndrome	78	59 (75.6)	3 (3.8)	7 (9.0)	9 (11.5)
Ataxia telangiectasia	150	69 (46.0)	16 (10.7)	32 (21.3)	33 (22.0)
Other immunodeficiencies	25	12 (48.0)	1 (4.0)	4 (16.0)	8 (32.0)
Total immunodeficiency categories	500	252 (50.4)	43 (8.6)	63 (16.0)	142 (28.4)

[a]Percentage of total tumors.
NHL, non-Hodgkin's lymphoma.

Table 53.9. Post-transplant lymphoproliferative disorders (reproduced from Harris *et al.*, 2001 with permission from IARC).

Categories of post-transplant lymphoproliferative diseases (PTLD)
Early lesions
Reactive plasmacytic hyperplasia
Infectious mononucleosis-like
Polymorphic PTLD
Monomorphic PTLD (classified according to the WHO nomenclature)
B-cell neoplasms
Diffuse large B-cell lymphoma (immunoblastic, centroblastic, anaplastic)
Burkitt's/Burkitt's-like lymphoma
Plasma cell myeloma
Plasmacytoma-like lesions
T-cell neoplasms
Peripheral T-cell lymphoma, not otherwise specified
Other types
Hodgkin's lymphoma and Hodgkin's lymphoma-like PTLD

In solid organ transplant recipients, PTLD is typically of recipient origin (>90%), whereas in hematopoietic stem cell transplant recipients it is most often of donor origin (Harris *et al.*, 2001a,b). The organ graft is frequently involved in PTLD (e.g., in 80% of lung transplanted, 33% of liver transplants, and 32% of kidney transplants in children) (Holmes and Sokol, 2002).

Epidemiology The epidemiology of PTLD remains scantily characterized. Occurrence varies by type of transplantation, ranging from a few percent or less in renal transplant recipients to ~20% in recipients of HLA mismatched, T-cell depleted bone marrow, and even 33% of child-recipients of combined liver-kidney transplants (Curtis *et al.*, 1999; Harris *et al.*, 2001a,b; Holmes and Sokol, 2002; Loren *et al.*, 2003).

PTLD may develop as soon as the first week or as late as 9 years after transplantation, but the median latency period is around 6 months in solid organ recipients and 70–90 days in hematopoietic stem cell recipients (Loren *et al.*, 2003). PTLD occurs in all age groups, but is seen in more men than women, which could, however, reflect gender differences in transplantation frequency (Hsu and Glaser, 2000). Inadequate T-cell control of EBV-infected B-lymphocytes is thought to be critical to the development of EBV-positive PTLDs. Consistent with this theory, established risk factors for PTLD include recipient EBV seronegativity (in particular if the donor is EBV-seropositive), primary or reactivated EBV infection following transplantation, high levels of immune suppression (cyclosporine, tacrolimus, antithymocyte globulin, or antilymphocyte antibodies), cytomegalovirus infection, transplanted organ (renal < non-renal) and – less certain – younger age (Aguilar *et al.*, 1999; Loren *et al.*, 2003; Swinnen, 2000).

Evidence of association with EBV The significance of EBV to PTLD development is implied first and foremost by demonstration of the virus in approximately 80% of cases (Harris *et al.*, 2001a,b). EBV-negative PTLDs have been described, and tend to occur with longer latency periods than their virus-positive counterparts (Nalesnik, 2002).

Because early recognition of developing PTLD may affect prognosis, attempts have been made to develop techniques

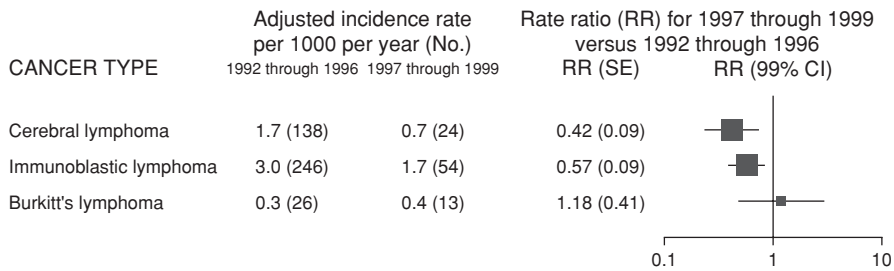

Fig. 53.11. Incidence rates of certain types of non-Hodgkin's lymphoma in 1992 through 1996 (before HAART) and in 1997 through 1999 [after HAART] and rate ratios (RRs) of incidence rates in 1992 through 1996 compared with 1997 through 1999. Reproduced from International Collaboration on HIV and Cancer 2000 with permission from Oxford University Press.

for monitoring EBV infection activity. Decreased anti-EBV nuclear antigen antibody levels have been associated with an increased risk for PTLD (Cen *et al.*, 1993; Riddler *et al.*, 1994). A more useful measure of EBV activity is, however, the assessment of the burden of virus infection in peripheral blood mononuclear cells and serum which, by a wide variety of different assays, have been found to correlate with risk for PTLD (Stevens *et al.*, 2002a,b).

Lymphomas in HIV

EBV infection is involved in different diseases in HIV-infected individuals, in particular malignant lymphomas and leiomyosarcomas.

Malignant lymphoma in people with HIV The risk for malignant lymphomas is increased massively in patients with acquired immune deficiency syndrome (AIDS). Accordingly, aggressive B-cell non-Hodgkin's lymphoma has been an AIDS-defining condition almost since the recognition of the HIV epidemic and is the second most common tumor associated with HIV (Dal Maso and Franceschi, 2003; Lim and Levine, 2005).

The magnitude of reported increases in risk for non-Hodgkin's lymphoma in persons with AIDS have varied somewhat, but generally have been in the order of 100-fold for all types of non-Hodgkin's lymphoma combined in the period before the introduction of Highly Active Anti-Retroviral Therapy (HAART) (Dal Maso and Franceschi, 2003), relative risks possibly being more increased in children (Biggar *et al.*, 2000) and less increased in elderly (Biggar *et al.*, 2004). Particularly elevated relative risks have been reported for high grade diffuse immunoblastic (652-fold increased) and Burkitt's lymphoma (261-fold increased) (Cote *et al.*, 1997). While some uncertainty has existed for Hodgkin's lymphoma, recent data have suggested that the risk for this lymphoma is also increased in HIV-infected persons, although to a much lesser extent, i.e., around 10-fold (Frisch *et al.*, 2001; IARC, 1996).

The risk of non-Hodgkin's lymphoma varies by level of immune suppression as measured by CD4 count and typically is a late manifestation of HIV infection (IARC, 1996). Consistent with this, the introduction of HAART seems to have accompanied by a decrease in AIDS-malignant lymphoma occurrence, notably of the subtypes most strongly associated with EBV (see below) (International Collaboration on HIV and Cancer, 2000; Lim and Levine, 2005) (Fig. 53.11).

In contrast to AIDS-related Kaposi's sarcoma, a human herpesvirus 8-associated malignancy that occurs predominantly in homo- or bisexual men, the occurrence of malignant lymphomas show no predilection for mode of HIV acquisition (IARC, 1996).

HIV itself appears not to be directly involved in the lymphoma development as illustrated by its absence in the malignant cells. Rather, through chronic antigenic stimulation causing B-cell proliferation, and through cytokine dysregulation, the virus may create an environment conducive for the development of malignant lymphomas (Levine, 2000; Knowles, 2001).

The range of different types of malignant lymphomas in HIV-infected persons is wide, but can be categorized according to whether they also occur in immune-competent individuals (Burkitt's lymphoma, diffuse large B-cell lymphoma, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue type (MALT lymphoma), peripheral T-cell lymphomas and classical Hodgkin's lymphoma), whether they are seen in other immune dysfunctional conditions (polymorphic B-cell lymphoma) or whether they are more specific to HIV infected individuals (primary effusion lymphoma, plasmablastic lymphoma of the oral cavity) (Raphael *et al.*, 2001). However, the various lymphoma subtypes are not equally frequent and a few of these make up the vast

majority of cases in AIDS, i.e., Burkitt's lymphoma (50%–60% of all HIV-related lymphomas) and diffuse large B-cell lymphomas (25% of all HIV-related lymphomas many of which present in the central nervous system), primary effusion lymphoma (less than 5% of all HIV-related lymphoma) and plasmablastic lymphoma of the oral cavity (Raphael *et al.*, 2001).

Evidence of association with EBV As for other lymphomas, the primary evidence of EBV involvement in development of HIV-related lymphomas is the demonstration of monoclonal virus in the tumors (Table 53.6). Overall, EBV is present in approximately 60% of HIV-related lymphomas, but the proportion of virus-positive tumors varies considerably with site of presentation and histological subtype. Thus, EBV is almost invariably present in Hodgkin's lymphomas, in (primary) central nervous system lymphomas and in primary effusion lymphomas (so-called PEL), in 80% of diffuse large cell lymphomas with immunoblastic features, in more than 50% of plasmablastic lymphomas of the oral cavity, in 30%–50% of AIDS-related Burkitt's lymphomas, and in 30% of diffuse large B-cell lymphomas of the centroblastic variant (Raphael *et al.*, 2001). As the risk of the various types of non-Hodgkin's lymphoma correlating with level of immune deficiency, a similar correlation arises with respect to prevalence of EBV in the lymphoma (Carbone and Gloghini, 2005).

Gastric carcinoma

Gastric carcinomas are tumors derived from the epithelium of the stomach mucosa with glandular differentiation (Fenoglio-Preiser *et al.*, 2000). Histologically, the tumors either are gland-forming with tubular, acinary, or papillary structures or display a complex mixture of dis-cohesive, isolated cells with variable morphologies, sometimes in combination with glandular, trabecular, or alveolar solid structures (Fenoglio-Preiser *et al.*, 2000). The current WHO classification of tumor of the digestive system distinguishes between tumors occurring at the junction between esophagus and the stomach, many of which would previously have been classified as cancers of the gastric cardia, and gastric cancer differing epidemiologically.

Epidemiology

The incidence of gastric cancer has been decreasing worldwide for several decades (Parkin *et al.*, 2002), yet with an estimated 876 000 incident cases annually it remains the fourth most common cancer worldwide, and because of its relatively poor prognosis the second most common cancer-specific cause of death attributed 647 000 deaths annually (Parkin *et al.*, 2000). There is considerable (more than 10-fold) geographical variation in the incidence of gastric cancer, with high rates in the Andean regions of South America, Eastern Europe, and Eastern Asia, and low rates in Northern Europe, most African countries, and North American whites (Fenoglio-Preiser *et al.*, 2006). For instance, world standardized incidence rates of 6.6 per 100 000 are observed in white American men as compared with rates between 60 and 90 per 100 000 observed in Japanese men (IARC, 2002). Irrespective of this geographical variation, the cancer is twice as common in men as in women (Parkin *et al.*, 2002; Nyren and Adami, 2002). In contrast to decreasing occurrence of gastric cancer, there is evidence to suggest that cancer of the esophageal–gastric junction may be increasing (Newnham *et al.*, 2003; Wijnhoven *et al.*, 2002), although this is still debated (Ekstrom *et al.*, 1999).

Both gastric cancer and cancers of the esophageal–gastric junction are rare before the age of 30 years, but increase with older age (Parkin *et al.*, 2002). Besides age, established risk factors for gastric cancer include familial occurrence, tobacco smoking, certain diets, alcohol, and infection with *Helicobacter pylori* (Nyren and Adami, 2002). With respect to familial occurrence of gastric carcinoma, it has been estimated that familial clustering of gastric cancer occurs in approximately 1% of patients (Shinmura *et al.*, 1999). Increased gastric cancer risk has been described in a number of hereditary conditions, notably the Li–Fraumeni syndrome and hereditary non-polyposis colorectal cancer, but other syndromes may exist (Nyren and Adami, 2002). Familial clustering of gastric cancer may reflect shared genetic or environmental factors, or both. Accordingly, in a study of more than 44 000 Scandinavian twins, compared to men whose twin did not have stomach cancer 7- and 10-fold increased risks for gastric cancer were observed in dizygotic and monozygotic male twins, whose twin had stomach cance. For women, the corresponding relative risk estimates were 6 and 20 respectively (Lichtenstein *et al.*, 2000). This corresponded to inherited factors accounting for 28%, shared environmental factors 10%, and non-shared environmental factors to 62% of the overall gastric cancer risk.

Smoking is associated with stomach cancer risk, in cohort studies amounting to 1.5 to 2-fold increased risk (Tredaniel *et al.*, 1997). Certain dietary items are suspected to be of importance for gastric cancer risk. Accordingly, salt intake and, in some populations, smoked or cured meats have been associated with increased risk of gastric cancer, whereas intake of fresh fruit and vegetables have been associated with a reduced gastric cancer risk (Nyren and Adami, 2002). Finally, several studies have shown that infection

with *Helicobacter pylori* carries an increased risk of gastric cancer. Thus, in a Japanese cohort study, 36 cases of gastric carcinoma was observed during follow-up in 1246 *Helicobacter pylori* infected persons compared with no cases among 280 uninfected men (Uemura *et al.*, 2001). Analogously, a meta-analysis of 42 studies showed *Helicobacter pylori* infection to be associated with a two-fold increased risk of gastric cancer (Eslick *et al.*, 1999).

Evidence of an association with EBV

The scientific evidence incriminating EBV in development of gastric carcinoma encompasses the demonstration of monoclonal virus in the malignant cells and aberrant anti-virus antibody patterns before and at diagnosis (Table 53.6).

The first suggestion of an involvement of EBV in gastric carcinoma development came in the early 1990s by the demonstration of the virus in lymphoepithelioma-like carcinomas of the stomach (Burke *et al.*, 1990). Subsequent investigations indicate that the virus is present in the vast majority of this specific subgroup of gastric carcinomas, i.e., 80+% (Fukayama *et al.*, 1998). Importantly, however, the association with EBV is not restricted to lymphoepithelioma-like gastric carcinomas as the virus can also be demonstrated in varying proportions (typically less than 10%) of carcinomas of more common histologies (Fukayama *et al.*, 1998; Imai *et al.*, 1994; Koriyama *et al.*, 2004; Shibata and Weiss, 1992; van Beek *et al.*, 2004).

EBV has also been implicated in gastric carcinoma development by serological studies. Specifically, elevated seroprevalence of anti-viral capsid antigen IgA and IgGy and elevated anti-early antigen antibies and (elevated IgG viral capsid antigen antibody titers) have been described at and before diagnosis of EBV-associated gastric carcinomas (Imai *et al.*, 1994; Levine *et al.*, 1995).

Besides, by histology the proportion of EBV-positive gastric carcinomas appears to vary by gender, age, tumor location, and possibly geography. EBV-positive gastric carcinomas seem to be more common in men than in women. In a large Japanese investigation of 1918 cases, EBV was demonstrated in 83 (6.8%) of 1212 male and in 17 (2.4%) of 706 female cases (Koriyama *et al.*, 2004). Likewise, in a recent Dutch series 38 (11.7%) of 324 gastric carcinomas in men and 3 (1.2%) of 242 carcinoma in women harbored EBV (van Beek *et al.*, 2004), and from smaller US series EBV prevalence of 15%–21% and 3%–5% are reported for gastric carcinomas in men and women, respectively (Shibata, 1998). Overall, the proportion of EBV-positive tumors seems to correlate inversely with age, but the age pattern may differ between gastric carcinoma subtypes (Koriyama *et al.*, 2004; van Beek *et al.*, 2004).

Interestingly, the EBV-positive tumors are not evenly distributed topographically in the stomach. Accordingly, the virus can more often be demonstrated in carcinomas of the cardia and the middle stomach than in the antrum, proportions varying 3–4 fold or more (Koriyama *et al.*, 2004; Takada, 2000; van Beek *et al.*, 2004). Of interest, EBV-positive tumors have been reported to make up a relatively large proportion of gastric carcinomas after partial gastrectomy for non-malignant diseases, published estimates ranging between 27% and 42% (Baas *et al.*, 1998; Chang *et al.*, 2000; Koriyama *et al.*, 2004; Nishikawa *et al.*, 2002; Yamamoto *et al.*, 1994). It has been suggested that this distribution of the EBV-positive tumors reflect that the non-neoplastic mucosa of the proximal stomach may be conditioned to develop EBV-related tumors (Fukayama *et al.*, 1998).

As illustrated in Fig. 53.12 the proportion of EBV-positive gastric carcinomas appears to differ between countries and may even vary within countries (Tashiro *et al.*, 1998). However, so far it has been difficult to define a clear geographic pattern in the distribution of EBV-related gastric carcinomas like those known for nasopharyngeal carcinoma and Burkitt's lymphoma. The precise siginificance of variation in referral patterns, case selection or possible variation in other risk factors for gastric carcinomas is difficult to evaluate in this context (Hsu and Glaser, 2000; Levin and Levine, 1998).

Lymphoepithelioma-like carcinomas may have a better prognosis than other gastric lymphomas, and recently, it has been suggested that other types of EBV-positive gastric carcinomas may also have better prognosis than virus-negative tumors (van Beek *et al.*, 2004).

Given the high incidence of gastric adenocarcinoma worldwide [around 870 000 incident cases annually (Parkin *et al.*, 2001), EBV-related gastric carcinoma, estimated to amount to at least 50 000 cases per year may be the most common of all EBV-associated malignancies (Takada, 2000) (Table 53.10).

Other malignancies associated with EBV infection

EBV has been shown, or is suspected, to be involved in the development of a series of other malignancies, much less frequently occurring than those described in the above sections. These include lymphoepithelioma-like carcinomas and soft tissue sarcomas, for which an association with EBV is compelling, and carcinomas of the breast, thymus, and liver, for which the evidence is less evident.

Lymphoepithelioma-like carcinomas

Lymphoepithelioma-like carcinomas are tumors that morphologically resemble undifferentiated carcinoma of the

Table 53.10. Estimated number of neoplasms associated with EBV (reproduced from Levin and Levine, 1998.)

Malignancy	Estimate of EBV genome positivity	Estimated number of cancers worldwide in 1990	Estimated number of EBV associated neoplasms
Gastric adenocarcinoma	6–12%	876 000	78 840
Nasopharyngeal cancer			
Developed countries	95%	5 500	5 225
Economically developing countries	99%	53 000	52 470
Hodgkin's lymphoma			
Developed countries	30–50%	25 000	10 000
Economically developing countries	60–90%	37 000	27 750
Non-Hodgkin's lymphomas	6–10%	223 000	17 840
Burkitt's lymphoma			
Developed countries	20–30%	600	7 440
Economically developing countries	95%	9 000	8 550
AIDS-related lymphoma	50–90%	9 000	6 300
Smooth muscle sarcomas	Case reports (rare malignancy)		
Lymphoepithelioma like carcinomas of the salivary gland, lung, stomach, thymus	Case reports (rare malignancies)		

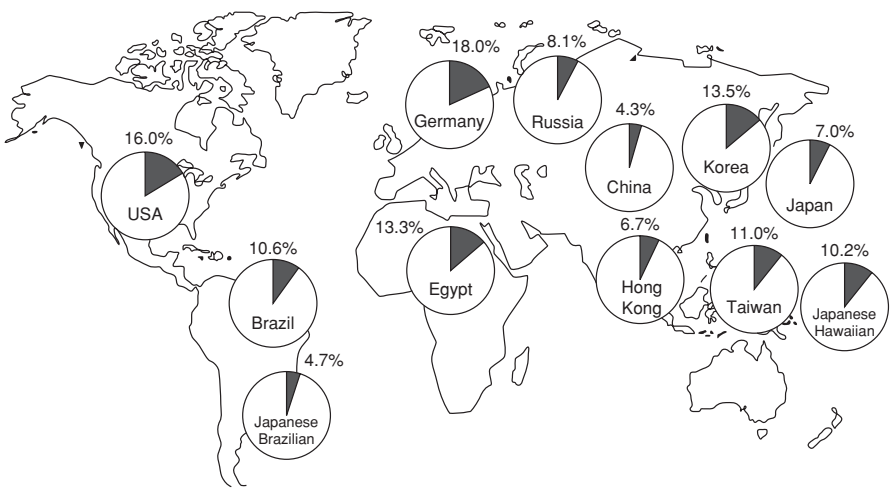

Fig. 53.12. World distribution of proportions of gastric cancers that are EBV-related. Reproduced from Tashiro *et al.*, 1998.

nasopharynx. In addition to the stomach (see above) the tumor has been described in the salivary glands, oral cavity, larynx, thymus, trachea, lungs, breast, uterine cervix, vagina, urinary bladder, skin, stomach (Iezzoni *et al.*, 1995), thyroid (Shek *et al.*, 1996), esophagus (Mori *et al.*, 1994), kidney (Elzevier *et al.*, 2002), ureter (Chalik *et al.*, 1998), and liver (Jeng *et al.*, 2001).

The tumor attracts interest because, in addition to the morphological similarity with nasopharyngeal carcinoma, EBV has been reportedly demonstrated in the malignant cells in some, but not all of the affected organs (Chalik *et al.*, 1998; Elzevier *et al.*, 2002; Iezzoni *et al.*, 1995; Jeng *et al.*, 2001; Mori *et al.*, 1994; Shek *et al.*, 1996).

Also, the prevalence of the virus in the malignant cells follows the same geographical distribution as observed nasopharyngeal carcinoma for salivary gland and lung lymphoepithelial-like carcinomas, being higher in Asian than in Western patients, thereby further underscoring the analogy between the two groups of tumors (Hsu and Glaser, 2000).

Leiomyosarcoma

Leiomyosarcomas are malignant neoplasms of smooth muscle tissues. They are exceedingly rare tumors, occurring at rates around 1 per 100 000, slightly higher in women than in men (Levi *et al.*, 1999; Zahm *et al.*, 1996) The connection with EBV is fairly recent and initially arose from the apparent accumulation in HIV infected children and organ transplant recipients (see for review Jenson, 2000 of cases reported until 1998). Epidemiologically, the association for HIV-infected children has been supported by surveys of cohorts of such children demonstrating excessive occurence of the tumor (Biggar *et al.*, 2000; Granovsky 1998). The conclusive piece of evidence for the association with EBV is, however, the demonstration of the virus in the tumors of immunocompromised hosts (Jenson, 2000).

Other malignancies

In recent years, the involvement of EBV in other malignancies than those discussed above has been debated. These include breast carcinomas (Glaser *et al.*, 2004a,b), hepatocellular carcinoma (Sugawara *et al.*, 1999), and thymomas (Chen, 2002). As yet, however, the evidence for the association with EBV remains controversial.

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54

The epidemiology of KSHV and its association with malignant disease

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Introduction

In 1872, Moritz Kaposi, a Hungarian dermatologist, described six patients with multifocal brown–red or blue–red nodules or plaques on the feet and hands (Kaposi, 1872). Initially called "Idiopathisches multiples Pigmentsarcoma der Haut" (multiple idiopathic sarcoma of the skin) by Kaposi, the condition later became known as Kaposi's sarcoma (KS). Decades later, after the epidemiology of KS began to be investigated, its uneven geographic distribution suggested that exogenous factors were etiologically important. Subsequently, as the AIDS epidemic unfolded in the early 1980s, homosexual men were found to be up to 20 times more likely than other risk groups to develop KS, a markedly disproportionate risk that led to the hypothesis that the exogenous factor was a sexually transmitted infectious agent (Beral *et al.*, 1990). Numerous microbial candidates were proposed (Drew *et al.*, 1982; Huang *et al.*, 1992; Wang *et al.*, 1993) but for none was convincing evidence demonstrated until Kaposi's sarcoma-associated herpesvirus (KSHV), or human herpesvirus 8 (HHV-8), was discovered in 1994 (Table 54.1) (Chang *et al.*, 1994). In a short period following the discovery of KSHV, consensus rapidly developed that it is a necessary, albeit not sufficient, causal agent of KS (Whitby *et al.*, 1995; Gao *et al.*, 1996a,b; Martin *et al.*, 1998; Renwick *et al.*,1998; O'Brien *et al.*, 1999). This discovery was more than academic in that, because of the AIDS epidemic, KS is now worldwide the fourth most common cancer caused by an infectious agent, following gastric, cervical, and hepatic cancer. Subsequently, it was also determined that KSHV has a potentially causal role in primary effusion lymphoma (PEL) (Cesarman *et al.*, 1995), also known as body cavity-based lymphoma, and multicentric Castleman's disease (Soulier *et al.*, 1995). Now that the clinical importance of KSHV has been established, attention has turned towards unraveling of what is proving to be a complicated epidemiologic profile for the virus.

Diagnosis of KSHV infection

While nucleic acid amplification techniques were originally used to identify the presence of KSHV DNA in KS tissue, when applied to peripheral blood these techniques are much less sensitive as compared to detection of KSHV-specific antibodies. Even among patients with KS, of whom virtually all have detectable KSHV DNA in their KS lesions, only approximately 40 to 60% have detectable KSHV DNA in either plasma or peripheral white blood cells (IARC Working Group, 1997). Among persons without KS, the group for which a diagnostic test for KSHV is most needed, the prevalence of KSHV DNA in blood is much lower, especially in HIV-uninfected populations, and is always less common than the detection of KSHV-specific antibodies. For this reason, there is currently little role for nucleic acid-based testing of peripheral blood in the diagnosis of KSHV infection (Spira *et al.*, 2000). Instead, the primary means of KSHV diagnosis is antibody testing, which is described in detail in Chapter 52. What follows is a brief overview of antibody testing that provides the requisite perspective for which to interpret epidemiologic studies of the distribution and transmission of KSHV infection.

Initial serologic assays for KSHV antibodies

After the finding that DNA from PEL biopsy specimens contains KSHV sequences (Cesarman *et al.*, 1995), the original assays for KSHV antibodies were developed using cell lines obtained from patients with PEL (Miller *et al.*, 1996; Kedes *et al.*, 1996; Gao *et al.*, 1996a,b; Simpson *et al.*, 1996) One of the first platforms were indirect immunofluorescence assays (IFAs) that utilized the cell lines in an

Table 54.1. Original detection of KSHV DNA by polymerase chain reaction in various tissue specimens

Tissue type	No. evaluated	No. (%) positive for KS330
KS, AIDS-related	27	25 (93)
Lymphoma, AIDS-related	27	3 (11)
Lymph nodes, AIDS-related	12	3 (25)
Lymphoma, non-AIDS	29	0
Lymph nodes, non-AIDS	7	0
Vascular tumors, HIV unknown	5	0
Opportunistic infections	13	0
Consecutive surgical biopsies, HIV unknown	49	0

From Chang *et al.* (1994).

uninduced state where KSHV is primarily in its latent phase. These assays detect antibodies to KSHV latency-associated nuclear antigen (LANA or LNA-1), the product of open reading frame (ORF) 73 (Rainbow *et al.*, 1997; Kedes *et al.*, 1997a,b). Extensive evaluation of these assays determined that anti-LANA seropositivity is found in approximately 80% of persons with KS and less than 5% of non-male-homosexual adults in the USA or Northern Europe (i.e., specificity of at least 95%) (Kedes *et al.*, 1996; Gao *et al.*, 1996a,b; Simpson *et al.*, 1996). Subsequently, it was determined that induction, using phorbol ester, of KSHV into a lytic phase resulted in IFAs with greater, albeit not complete, sensitivity (approximately 95%) in terms of identifying KS patients, while maintaining seropositivity of less than 10% in non-homosexual adults in the USA (Chandran *et al.*, 1998). In an attempt to improve throughput and eliminate the subjective interpretation inherent in IFAs, a variety of other first-generation assays with comparable performance characteristics were developed. These include enzyme immunoassays (EIA) detecting antibodies against (a) bacterially derived recombinant portions of a minor capsid protein (ORF 65) (Simpson *et al.*, 1996), LANA (Renwick *et al.*, 1998), and glycoprotein K8.1 (Raab *et al.*, 1998; Engels *et al.*, 2000); (b) synthetic peptides portions of K8.1 and ORF 65 (Spira *et al.*, 2000; Pau *et al.*, 1998); and (c) whole virus (Chatlynne *et al.*, 2001; Martin *et al.*, 2000).

Methodologic challenges in serologic assay development

Although first-generation antibody assays, particularly those detecting anti-LANA antibodies, have been useful in epidemiologic work, no single first-generation serologic assay has demonstrated very high sensitivity and specificity, and agreement between assays has been suboptimal. In a study of seven first-generation antibody assays examining 143 serum specimens, the percentage of KSHV-seropositive specimens determined by the different assays ranged from 0% to 54% for the subset of specimens from blood donors, 27% to 60% for HIV-infected persons without KS, and 49% to 93% for KS patients (Rabkin *et al.*, 1998). More recently, a comparison of KSHV antibody testing from six laboratories, using newly developed assays or second-generation versions of previously examined assays, found improved agreement when testing serum specimens from persons with KS (all six laboratories found all 21 specimens to be positive) but only modest agreement when evaluating 1000 specimens from blood donors (a low risk group for KSHV infection) (Pellett *et al.*, 2003).

In part, the interassay disagreement that has been observed may be because certain assays target different antibodies for which inherent sensitivity and specificity for KSHV infection may differ (e.g., antibodies against lytic-phase versus latent-phase antigens). In other instances, however, assay calibration (i.e., differentiating positive from negative results) has not been done in a standardized fashion across assays with reference to a wide spectrum of gold standard KSHV-infected (true positive) and KSHV-uninfected (true negative) subjects. Determining assay sensitivity by limiting the true positive reference group to persons with KS, as has been typically done in the initially described assays, may result in estimates of sensitivity that are unrealistically inflated. This is because it is now recognized that, among KSHV-infected persons, antibody titer is highest and therefore easier to detect in KS patients (Gao *et al.*, 1996a,b). One attempt to reduce this "spectrum bias" (Ransohoff and Feinstein, 1978) was by Engels *et al.*, who used diluted serum from KS patients in an attempt to mimic KSHV-infected persons without KS and found rapidly diminishing sensitivity with only minimal dilution (Engels *et al.*, 2000). However, how much dilution is needed to mimic infected but non-diseased persons is not known. Another attempt was the use of homosexual men without KS but who had detectable KSHV in their saliva (Casper *et al.*, 2002a,b). These persons, however, may be enriched for antibody-positivity if the presence of KSHV antigen drives antibody production. Therefore, more work is needed in identifying gold standard KSHV-infected reference subjects without KS for the evaluation of assay sensitivity.

While early serologic investigators at a minimum had the presence of KS as a basis for forming a true positive reference group, assembling a true negative (KSHV-uninfected) reference group has been particularly vexing because of the lack of any analogous gold standard certifying absence of

infection. For example, the use of blood donors (Simpson *et al.*, 1996; Pau *et al.*, 1998; Rabkin *et al.*, 1998; Tedeschi *et al.*, 1999) is problematic because they may include men with undisclosed homosexual activity, the primary risk factor for KSHV infection in developed countries (described below). Approaches used in more recent work to optimize the assembly of a true negative reference group have capitalized on what is now known about the epidemiology of KSHV infection and of KS *per se*. In the USA, for example, this includes the use of virginal women and of young children who are just past the age of harboring maternal antibodies (Martin *et al.*, 2000), HIV-infected hemophiliacs and their female sexual partners, both of whom were known not to subsequently develop KS after a substantial period of observation (Engels *et al.*, 2000), and homosexual women (Casper *et al.*, 2002a,b).

In addition to the uncertainties in assembling the reference populations for assay calibration, other technical differences have likely contributed to poor interassay agreement. For example, the method of cut-off generation for a positive versus negative result has varied considerably. Some investigators have set cut-offs *a priori* by taking the mean of between 5 and 40 individuals considered to be uninfected and then adding two to five standard deviations (Renwick *et al.*, 1998; Simpson *et al.*, 1996; Pau *et al.*, 1998; Chatlynne *et al.*, 2001; Rabkin *et al.*, 1998; Tedeschi *et al.*, 1999; Davis *et al.*, 1997). The small sample sizes used in these calculations and the wide range of standard deviations employed likely result in substantial variation across assays. An improved approach to cut-off generation has been used in more recent work where receiver-operating characteristic (ROC) curves were described (Engels *et al.*, 2000; Martin *et al.*, 2000; Casper *et al.*, 2002; Corchero *et al.*, 2001; Laney *et al.*, 2006). ROC curves are not dependent upon an *a priori* cut-off, but instead show the paired sensitivity and specificity estimates associated with various empiric cut-offs. However, what has not yet been performed is an assessment of interassay agreement by having each assay examine sera from a panel of well-conceived true negative and true positive subjects and then compare ROC curves, generated in a standard fashion for each assay. Until this is done, it will not be possible to compare agreement between contemporary assays.

Approaches using a combination of antibody assays

An approach featuring a highly sensitive high-throughput serologic screening assay followed by a more labor-intensive confirmatory assay with both very high sensitivity and specificity, akin to what is done for the diagnosis of HIV infection, cannot yet be performed for KSHV infection because of the lack of a such confirmatory assay. Nonetheless, use of a combination of assays has been investigated in an attempt to improve assay accuracy compared to individual assays used in isolation (Engels *et al.*, 2003; Casper *et al.*, 2002; Laney *et al.*, 2006). In the most comprehensive analysis of assay combinations, joint use of EIAs featuring K8.1, ORF 73, ORF 65, and whole virus lysate antigens and a latent-phase IFA only marginally improved sensitivity (while holding specificity constant) compared to the best performing individual assays (Engels *et al.*, 2000). Rather than parallel use of multiple assays, the same degree of improvement in accuracy can be obtained when assays are used serially, with the use of a high-throughput EIA first, reserving the more time-intensive IFA in an attempt to resolve specimens scoring in the indeterminate range in the EIA (Engels *et al.*, 2000). Not only is this more cost-efficient than simultaneous testing with both assays, the improvement in accuracy can be substantial depending upon the initial EIA used (Casper *et al.*, 2002a,b).

Recently developed eukaryotically derived recombinant antigen-based serologic assays

While the use of induced PEL cell IFAs increases assay sensitivity, the lack of a KSHV-uninfected isogeneic control cell line hinders the discrimination of KSHV-specific antibody binding vs. non-specific binding to cellular components. Furthermore, because PEL cell IFAs express most or all of the KSHV-encoded genes, including those conserved across all herpesviruses, there is the threat of cross-reactivity with other herpesviruses. To address this, Inoue *et al.* used a mammalian expression system with BHK-21 cells infected with recombinant Semliki Forest virus (SFV) carrying individual KSHV proteins that are unique to KSHV (Inoue *et al.*, 2000). This approach has the advantage of having an isogeneic control, very high antigen expression, and, because it is a mammalian system, protein glycosylation, which may be an important component of epitope antigenicity. To date, separate BHK-21-based IFAs expressing ORF K8.1, ORF 73, and ORF 65 have been established. (Inoue *et al.*, 2000; Quinlivan *et al.*, 2001). Among these, the K8.1SFV IFA has exhibited the best performance. Among 72 KS patients, the K8.1SFV IFA detected antibodies in 94.4%, and among 30 blood donors, 2 (6.7%) were reactive, thus indicating an estimated specificity of at least 93.3%. These approaches will offer greatest utility when they are available in high throughput formats, and in this regard, Laney *et al.* have recently developed an EIA featuring a baculovirus-insect cell system-derived full length LANA protein as the antigen substrate (Laney *et al.*, 2006).

Seroreversion

Further complicating the interpretation of serologic testing is the phenomenon of a negative result following a positive result, termed seroreversion, noted in longitudinal observation of individual subjects. For example, Quinlivan *et al.* found that 27 (82%) of 33 subjects who had at least one time point seropositive for antibodies to LANA had at least one subsequent time point exhibiting seroreversion (Quinlivan *et al.*, 2001). This has also been reported by others (Chohan *et al.*, 2004 and Minhas *et al.*, 2006). Whether seroreversion is the result of assay measurement error (i.e., inadequate reproducibility) or whether it reflects true biologic diminution or loss of antibody is not known. In any case, its presence clouds the interpretation of a single seronegative serologic test result in a cross-sectional study.

Differential reactivity to KSHV antigens over time

Related to the idea that biologic phenomena may be responsible for seroreversion is the finding that KSHV-infected persons may develop antibodies to different antigens at different points in time. There are few longitudinal studies available, especially those with adequate lead-in periods that first establish seronegativity, but in work that has been done with antigen-specific assays, it appears that seroconversion to different antigens can occur at different times (Quinlivan *et al.*, 2001; Biggar *et al.*, 2003; Spira *et al.*, 2001). This further highlights how the currently available assays targeting single antigens are likely not optimally sensitive for detecting infection.

Utility of currently available serologic assays

For epidemiologic work where between group comparisons are the primary focus, even using first-generation serologic assays, despite their poor inter-laboratory agreement, has resulted in reproducible and plausible inferences (described below). Use of currently available serologic assays for individual-level diagnosis, however, be it in an epidemiologic study in the determination of seroconversion among individual subjects, or in the clinical setting, requires greater caution. The lack of gold standards in the development of assays, the apparent lack of concurrent high-level sensitivity and specificity among those first-generation assays that have been evaluated most extensively, and the phenomenon of seroreversion make definitive diagnosis of the presence or absence of KSHV infection problematic at an individual level. The only exception is persons who test positive on a very specific assay, such as the anti-LANA IFA (at least as performed in experienced laboratories). Currently, there is no Food and Drug Administration-licensed assay for KSHV antibodies; testing is limited to research laboratories. Clinicians who seek to use the avaible assays should be aware of their pitfalls and knowledgeable about their performance characteristics. Finally, the recent success in developing an essentially 100% sensitive and specific serologic assay for HIV infection, has raised expectations for the serologic detection of all newly described pathogens, including KSHV. Although not directly studied, the sensitivity and specificity of currently available KSHV antibody assays may not appreciably differ from antibody assays for other herpesviruses, none of which by virtue of their earlier discovery was investigated with the serologic expectations of the contemporary era. Whether or not the recently developed eucaryotically-derived recombinant antigen-based assays will demonstrate very high and reproducible specificity and sensitivity upon wide scale testing or whether there exist inherent biologic limitations to the serologic detection of KSHV infection remains to be determined.

Epidemiology of KSHV infection

Prevalence of infection Geographic distribution

Extensive worldwide surveys of KSHV seroprevalence using the same serologic assay and same method of sampling of subjects (e.g., similar age groups) have not been performed. Even with this limitation, three major patterns of seroprevalence have reproducibly emerged: high-level endemic, intermediate-level endemic, and non-endemic. High-level endemic areas are defined by those with seroprevalences between 30% and 70% among general adult populations and are found in many parts of Africa (Olsen *et al.*, 1998; Mayama *et al.*, 1998; Gessain *et al.*, 1999) and the Middle East, e.g., Egypt (Andreoni *et al.*, 1999). As is discussed below, it is the catastrophic intersection between the endemic nature of KSHV and the HIV epidemic in sub-Saharan Africa that has resulted in KS becoming the most common adult malignancy in many areas there (Chokunonga *et al.*, 2000; Wabinga *et al.*, 2000).

However, even within these geographically constrained areas there nonetheless exists important differences in KSHV seroprevalence from region to region. For example, recent work using a standardized approach to antibody testing found considerably higher seroprevalence in Uganda than compared to South Africa (Butler *et al.*, 2006).

Intermediate-level endemic areas feature KSHV seroprevalences between 10 and 25% in the general population and are found primarily in the Mediterranean area (Angeloni *et al.*, 1998; Whitby *et al.*, 1998; Cattani *et al.*, 2003) Again even within this defined geographic area, there

is considerable heterogeneity. For example, among blood donors in Italy, prevalence was estimated to be 7.3% in Northern and Central Italy and 24.6% in Southern Italy (Whitby *et al.*, 1998).

Non-endemic areas are those with seroprevalences less than 10% in the general population. These include North America, Central America, South America, Northern Europe, and Asia. In these non-endemic areas, however, certain population groups have seroprevalences that rival those in high and intermediate-level prevalence regions. In particular, it is homosexual men across these non-endemic areas that have consistently been shown to have the highest seroprevalence; between 30% and 60% of HIV-infected homosexual men in these areas and 20% and 30% of HIV-uninfected homosexual men are KSHV-infected (Martin *et al.*, 1998; O'Brien *et al.*, 1999; Kedes *et al.*, 1996; Chandran *et al.*, 1998; Melbye *et al.*, 1998; Dukers *et al.*, 2000). This contrasts with less than 10% prevalence in most reports of women and non-homosexual men in the general population of these areas (Martin *et al.*, 1998; Kedes *et al.*, 1996; Smith *et al.*, 1999; Kedes *et al.*, 1997a,b). Other notable pockets of concentrated prevalence (not all of which have been confirmed by other investigators) in otherwise non-endemic areas include an estimated seroprevalence of 65% among Brazilian Amerindians >30 years of age (Biggar *et al.*, 2000), 33% among adults in Papua New Guinea (Rezza *et al.*, 2001), 20% among adults of the Noir-Marron, an ethnic group of African origin residing in French Guiana (Plancoulaine *et al.*, 2000), and 25% among Hispanic children in South Texas in the USA (Baillargeon *et al.*, 2002). Although considerable work has now firmly established the marked geographic differences in KSHV seroprevalence, it is notable (as will be discussed below) that the explanation for this is unknown. Whether the answer rests in host genetic or behavioral differences, influences from other exogenous environmental influences, or viral variants is unresolved.

Temporal patterns

Studies of genomic strain variability have concluded that KSHV is an ancient human virus, introduced at least tens of thousands of years ago (Hayward, 1999) (and discussed below). Descriptions of KS in the 1800s and the finding of high KSHV seroprevalences in geographically distinct and remote isolated populations (Biggar *et al.*, 2000; Rezza *et al.*, 2001; Whitby *et al.*, 2004) substantiate this. There is, however, debate as to the introduction of KSHV in sentinel populations such as homosexual men in the USA and Northern Europe. Two initial reports hypothesized that an epidemic of KSHV infection in homosexual men took place concurrently with that of HIV infection (O'Brien *et al.*, 1999; Melbye *et al.*, 1998). This hypothesis was based upon the finding of an initial high incidence of KSHV infection in these studies at the beginning of their observation in 1981 and 1982, respectively. However, a more direct examination of homosexual men in San Francisco as early as 1978, prior to the HIV epidemic, found that seroprevalence was already 24.9% (Osmond *et al.*, 2002). An endemic state of KSHV infection in homosexual men has also been suggested in Denmark in the 1970s, both in a study directly measuring KSHV seroprevalence (Hjalgrim *et al.*, 2001) and in a study of KS which found that never-married men (a crude surrogate for homosexual men) were significantly at risk (Hjalgrim *et al.*, 1996). Therefore, the introduction of KSHV infection appears to have substantially preceded that of HIV, although how long KSHV has been transmitted at high rates among homosexual men is not known.

There is little information on the patterns of KSHV infection in different populations over time. In Africa, among samples collected between 1972 and 1978 in Uganda and Tanzania, the seroprevalence of KSHV infection was similar to that found in the 1980s and 1990s, suggesting there has been little influence from the emerging HIV epidemic (de-The *et al.*, 1999). In homosexual men in developed countries, there had been speculation, based upon the report of either decreased incidence of AIDS-associated KS or a decline in KS as a proportion of new AIDS cases, that the prevalence of KSHV infection had fallen in the late 1980s and early 1990s (Rutherford *et al.*, 1990; Dore *et al.*, 1996; Jones *et al.*, 1999). However, a decline in the overall KS incidence rate could have been caused by reduced HIV transmission alone, resulting in fewer immunocompromised persons available to develop KS. Similarly, a decline in KS as a proportion of new AIDS cases could be caused by a decline in the proportion of new cases occurring in homosexual men since KS is uncommon in risk groups other than homosexual men. Direct examination of KSHV seroprevalence in homosexual men in San Francisco (including both HIV-infected and uninfected men) found a stable prevalence between 26% and 29% in the periods spanning 1978–80, 1984–85, and 1995–96 (Osmond *et al.*, 2002). Similarly, KSHV seroprevalence was stable at approximately 60% in the period spanning 1985 to 1995 among homosexual men in Italy who had recently acquired HIV infection (Rezza *et al.*, 2000a,b).

Genotypic diversity of infection

The entire KSHV genome was sequenced shortly after its detection (Russo *et al.*, 1996; Neipel *et al.*, 1997), and considerable work thereafter has documented extensive genotypic heterogeneity across populations. Initial work

examining genotypic diversity focused on segments of ORF 26 and ORF 75, and although there was not extensive overall variation (only 1.5% of nucleotide positions), there was enough clustering to form three distinct but narrow subtypes in the initial 12 specimens examined (Zong *et al.*, 1997). Subsequently, ORF K1, K12, 73, K14.1, and K15 have also been examined in many more specimens, resulting in the finding of significantly more genotypic heterogeneity and a much more complicated interpretation. Of these regions, the far left hand (ORF K1) and right hand (ORF K15) sides of the genome are most interesting. ORF K1 is the most heterogenous, with up to 44% amino acid differences across strains, allowing for the formation of at least five major subtypes and at least 24 subgroups, many of which indicate intertype recombination (Biggar *et al.*, 2000; Nicholas *et al.*, 1998; Zong *et al.*, 1999; Cook *et al.*, 1999; Meng *et al.*, 1999; Zong *et al.*, 2002). This degree of diversity has been seen with relatively few isolates examined thus far (less than 250); as more are sequenced, it is apparent that even more distinct subtypes will be identified (Whitby *et al.*, 2004). ORF K15 features two highly diverged alleles, termed P (for predominant) and M (for minor) corresponding to their prevalence in isolates examined to date, which have only 33% amino acid homology to one another (Poole *et al.*, 1999). The two alleles are so disparate that it has been suggested the minor form was introduced via a recombination event with a related but as yet undocumented primate virus (Poole *et al.*, 1999).

While there is an impressive degree of between-subject genotypic diversity, the data on within-subject heterogeneity are conflicting. The majority of initial reports examining sequence variability within individuals either at one time point with multiple samplings or over time did not find evidence of strain (Meng *et al.*, 1999; Zong *et al.*, 1999; Stebbing *et al.*, 2001; Codish *et al.*, 2000), and even among those that did (Gao *et al.*, 1999; Lacoste *et al.*, 2000a,b), intrahost variability was very low. More recently, however, work examining three different types of oral fluid collection, as well as blood, and using a high-fidelity PCR approach, found that among 24 Malawians, 16 had evidence of strain variability in ORF K1 (Beyari *et al.*, 2003). Seven of the subjects harbored two or more subtypes. The reasons for the discrepancy between this and prior work is not clear, but if these findings are confirmed, they will demand significant caution in performing and interpreting future molecular epidemiology studies, particularly those focusing on interpersonal transmission.

Nomenclature schemes for the genotypic diversity of KSHV are still in evolution and not yet standardized between authors. Based on ORF K1 typing alone, at least three approaches have been proposed. Zong *et al.* (1997) proposed naming subtypes A–D, with numerical subgroups within subtypes (e.g., A1–A5′). Cook *et al.* (1999) proposed subtypes A–C with two forms of subgroups, one denoted with numbers (e.g., A3 and A5) and another with superscripts (e.g., A′). Finally, Meng *et al.* (1999) proposed genotypes I–IV with subtypes (e.g., I–A to I–F). To each of these schemes, a fifth major subtype (termed subtype E) was subsequently described among remote isolated South American populations (Biggar *et al.*, 2000; Whitby *et al.*, 2004). Overall genotypic classification is even more complicated because there is not complete linkage in strain diversity across the genome. For example, diversity in ORF K15, although limited to the P vs. M nomenclature, is largely independent of ORF K1 genotype (Zong *et al.*, 2002; Lacoste *et al.*, 2000a,b). When the internal coding regions (e.g., ORF 26, K12, 73, 75) are also considered, there becomes the need to describe at least three or four segments to fully describe a strain (e.g., as C3/A/A2/M) (Zong *et al.*, 2002).

Despite the complexity in nomenclature, there do appear to be emerging patterns in the geographic distribution of the different subtypes. Following the classification scheme proposed by Zong *et al.* for ORF K1 (Zong *et al.*, 1997, 2002), it has been observed that B subtypes are found almost exclusively in sub-Saharan Africa, D subtypes in South Asia/Australia/Pacific, and A and C subtypes in the U.S./Europe/North Asia (with a notable exception being the presence of A5 subtypes in Africa). This has led to the speculation that KSHV was present in the origins of modern humans in Africa (B subtype), and that the separation into the three main branches (A/C, B, and D) is explained by isolation and founder effects associated with the original migrations of human out of Africa, first to the Middle East, and then South Asia (D subtype), and later to Europe, North Asia, and the Americas (A and C subtypes) (Hayward, 1999; Zong *et al.*, 2002). Notable examples of this geographic association are seen in immigrants whose KSHV sequences match those of their countries of origin rather than their adopted residence (Zong *et al.*, 2002). The story is not entirely consistent, however, because of the unexpected finding of subtype E in isolated South American populations (Biggar *et al.*, 2000; Whitby *et al.*, 2004). Whereas it would have been expected that novel subtypes in South America would be most closely related to subtypes A and C (or even B), subtype E is most closely related to subtype D. Finally, the finding of M allele types for ORF K15 in ORF K1 A, B, or C strains further complicates the explanation regarding the geographic spread of the virus over time.

Aside from tracing the spread of KSHV over time and across human populations, the other implications of the substantial genotypic diversity are not well understood.

Table 54.2. Current strength of evidence[a] for various routes of KSHV transmission in endemic vs. non-endemic areas

Route of transmission	Endemic areas	Non-endemic areas
Sexual transmission among homosexual men	++	+++
Sexual transmission among heterosexual men and women	++	+
Non-sexual horizontal transmission in childhood	+++	n.s.
Injection drug use	n.s.	+++
Vertical transmission	+	n.s.
Blood transfusion	+++	+
Solid organ transplantation	+++	+++

[a] + denotes weak evidence; ++ denotes moderate evidence; +++ denotes definitive or strong evidence; – denotes presence of studies but no supportive evidence; n.s. denotes not yet studied.

First, while it is likely that the considerable heterogeneity in ORF K1 is providing clues regarding a powerful biological selection process, whether this involves immune evasion or some other mechanism is not known. Second, it is not known whether genotype plays a role in the ease of transmissibility and acquisition of KSHV. For example, it has not been established whether the localization of the B subtype in Africa can explain the high KSHV seroprevalence there compared to other regions. If genotype is important in viral transmission, high transmissibility is likely not limited to subtype B, as E subtypes in remote South American populations are also associated with very high community seroprevalences (Biggar *et al.*, 2000; Whitby *et al.*, 2004). Third, there has thus far been no convincing evidence for the importance of genotype in either the development of KSHV-related disease per se, the type of KSHV-related disease manifestation (KS vs. primary effusion lymphoma vs multicentric Castleman's diseases), or clinical severity once disease occurs. Of these potential questions, data are most abundant, but not yet definitive, on the issue of association with type of disease where work to date finds no correlation (Zong *et al.*, 2002; Lacoste *et al.*, 2000a,b). One report found an association between single nucleotide polymorphisms (SNPs) at nucleotide positions 1032 and 1055 of ORF 26, but this was among many SNPs evaluated and requires confirmation (Endo *et al.*, 2003). The lack of evidence for the importance of genotype in these various questions should not be interpreted as no role for genotype, but rather a lack of definitive data owing primarily to study designs that have focused on convenience samples of virus obtained from diseased patients (most of whom have a poorly characterized clinical course), rather than systematic sampling of asymptomatically-infected individuals (Whitby *et al.*, 2004) and well-characterized diseased individuals. The current inability to detect and sequence KSHV DNA from all KSHV-infected individuals (as defined by antibody-positivity) is a substantial obstacle to addressing these questions.

Routes of transmission

Current knowledge about KSHV transmission can be summarized as follows (Table 54.2). There is definitive evidence that it is transmitted by some form of intimate contact between homosexual men and equally persuasive evidence that it is transmitted in childhood by a horizontal non-sexual route in high-level endemic areas. There is also strong evidence that it is transmitted by infected organs in transplantation, by sharing of equipment among injection drug users, and by transfusion of whole unprocessed blood in high-level endemic areas. What is not known are the specific sexual behaviors that transmit KSHV among homosexual men, the exact means of horizontal spread to children in endemic areas, whether KSHV is transmitted vertically, and whether transmission occurs via transfusion of blood products that have undergone conventional processing techniques.

Transmission in non-endemic areas

Sexual transmission

Initial seroepidemiologic studies postulated that KSHV was sexually transmitted by showing that, other than in KS patients, seroprevalence was highest in homosexual men and other groups at highest risk for sexually transmitted disease (STD). For example, Kedes *et al.* (1996) using an IFA for antibodies directed against LANA, found among STD clinic clients in San Francisco that 6% of heterosexuals, 13% of HIV-uninfected homosexual men, and 35% of HIV-infected homosexual men were seropositive; seroprevalence in screened blood donors was 1%. Reports of this type, however, describing an association between risk of STD and KSHV seroprevalence, provide only indirect evidence for sexual transmission. More definitive evidence is found in studies with direct measurement of sexual activity, controlled for other potential routes of spread. Among a population-based sample of homosexual men in San Francisco, KSHV seroprevalence increased linearly with the number of male intercourse partners in the previous two years (Fig. 54.1) (Martin *et al.*, 1998). This association was independent of recreational drug use, transfusion history, CD4 lymphocyte count, and HIV serostatus. The strength of this association, confirmed by others (O'Brien *et al.*, 1999;

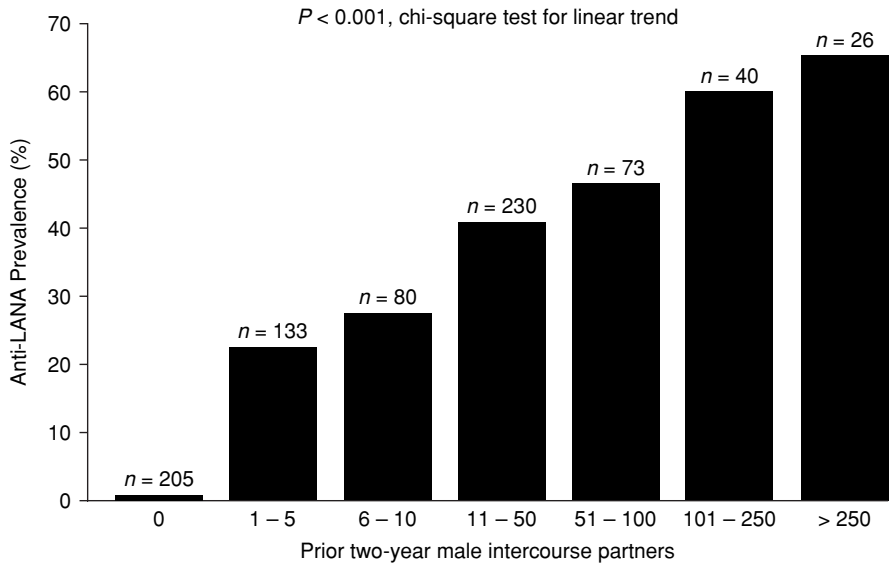

Fig. 54.1. Prevalence of antibodies to KSHV latency-associated nuclear antigen (LANA) by number of prior two-year male intercourse partners among men in San Francisco. Number atop each column signifies number of subjects in each group. From Martin *et al.*, 1998.

Melbye *et al.*, 1998; Dukers *et al.*, 2000; Pauk *et al.*, 2000; Jacobson *et al.*, 2000a,b; Diamond *et al.*, 2001; Casper *et al.*, 2002a,b), leaves little doubt that some form of intimate contact transmits the virus among homosexual men. However, because "sexual partner" is defined broadly in most of these reports to include a variety of practices (e.g., insertive and receptive penile-anal intercourse, penile–oral intercourse, and oral–anal contact), these data alone do not pinpoint the specific route of sexual transmission.

Even prior to the discovery of KSHV, many studies had sought to identify the specific route of transmission for the putative KS agent. Some (Jacobson *et al.*, 1990; Beral *et al.*, 1992; Grulich *et al.*, 1997), but not all (Archibald *et al.*, 1990; Lifson *et al.*, 1990; Page-Bodkin *et al.*, 1992; Elford *et al.*, 1992) found that insertive oral–anal sexual practices ("rimming") were a risk factor for KS and, by extension, were a risk factor for the as-yet-undiscovered KS agent. The discovery of KSHV was hoped to bring clarity to this question, but initial studies among homosexual men have been conflicting, variously reporting the strongest association for penile–anal intercourse, penile–oral intercourse, oral-anal contact, or kissing. To date, at least 8 studies have evaluated specific practices (Table 54.3) (O'Brien *et al.*, 1999; Melbye *et al.*, 1998; Dukers *et al.*, 2000; Pauk *et al.*, 2000; Jacobson *et al.*, 2000a,b; Diamond *et al.*, 2001; Casper *et al.*, 2002a,b; Grulich *et al.*, 1999). For almost all of the acts, at least one study has reported an association. However, for no act have all (or nearly all) studies found an association. Receptive penile-anal intercourse, the act that has attracted the most support, is paradoxically one in which the apparent culprit body fluid, semen, is now known to rarely harbor KSHV (Pauk *et al.*, 2000; Diamond *et al.*, 1997; Koelle *et al.*, 1997).

There are numerous potential biases, reviewed elsewhere (Martin and Osmond, 2000), inherent in the work to date that might explain the inconsistency among studies evaluating specific routes of KSHV sexual transmission. One potential bias is inaccuracy in measuring exposure, in this case the self-report of sexual acts, which is complicated in cross-sectional studies by not knowing the relevant period of exposure to query. A second problem, deficiencies in sensitivity and/or specificity of KSHV serologic assays, particularly with the first-generation assays used to date, results in both misclassification of who is infected and mistaken ascertainment of sexual practices in longitudinal studies by querying for sexual behavior during the incorrect time period. Finally, because sexual acts are correlated in homosexual men, failure to adjust for all of the relevant acts can lead to confounding. In other words, finding an association for a particular act may simply be because that act is correlated with another act that is the true culprit in transmission. All but one (Pauk *et al.*, 2000) of the studies to date were assembled to study HIV infection, and hence in general did not record acts, such as kissing, where saliva is passed. Moreover, no study recorded information on less obvious uses of saliva in various sexual acts such as a lubricant for penile-anal intercourse or when a finger is inserted into an anus (Butler *et al.*, 2003).

Table 54.3. Studies evaluating specific sexual practices as routes of KSHV transmission in homosexual men

		Specific Sexual Practice						
		Penile–anal intercourse		Penile–oral intercourse		Oral-anal contact		
Study	Design	Ins.[a]	Rec.[a]	Ins.	Rec.	Ins.	Rec.	Kissing
Melbye, 1998 (Melbye *et al.*, 1998)	cohort	ne[c]	+[b]	ne	−[b]	−	−	−
O'Brien, 1999 (O'Brien *et al.*, 1999)	cohort	+	+	−	−	+	−	ne
Grulich, 1999 (Grulich *et al.*, 1999)	cross-sectional	−	−	−	−	−	−	ne
Dukers, 2000 (Dukers *et al.*, 2000)	cohort	−	−	+	+	−	−	ne
Jacobson, 2000 (Jacobson *et al.*, 2000)	cohort	ne	+	−	−	ne	ne	ne
Pauk, 2000 (Pauk *et al.*, 2000)	cross-sectional	ne	ne	ne	ne	ne	ne	+
Diamond, 2001 (Diamond *et al.*, 2001)	cross-sectional	−	+	ne	ne	ne	ne	ne
Casper, 2002 (Casper *et al.*, 2002a,b)	cross-sect/cohort	−	−	ne	ne	−	−	ne

[a] "Ins." denotes insertive practice of the act; "Rec." denotes receptive practice of the act
[b] "+" indicates an association was found; "−" indicates no association
[c] "ne" denotes the act was not evaluated

Recording of acts where saliva is exchanged is important because, unlike semen, saliva is now known to be the body fluid that most commonly harbors KSHV (Pauk *et al.*, 2000; Koelle *et al.*, 1997). Careful work from Seattle, for example, found that among KSHV-seropositive homosexual men, 30% of oral samples contained KSHV DNA, vs. 1% of genital and anal samples (Pauk *et al.*, 2000). Therefore, failure to record all of the relevant acts may result both in the inability to find the true culprit act of transmission and the spurious assertion of the importance of a correlated but non-transmitting act.

Recognition that saliva is the body fluid that most commonly harbors KSHV has naturally focused attention on transmission by kissing (Pauk *et al.*, 2000). However, because kissing is common in all population groups, the low prevalence of KSHV in the general population argues against kissing as a dominant route of spread. Indeed, other herpesviruses transmitted by kissing (e.g., EBV) are highly prevalent in the general population. One scenario where kissing could be the major route of spread but still not result in high prevalence in the general population is if KSHV was recently introduced into homosexual men and has not yet spread to others. Data from San Francisco, however, show that KSHV was not recently introduced into homosexual men. Seroprevalence among homosexual men in San Francisco in 1978–80 was estimated to already be high at 28.4% and stable over the next 15 years (26.4% in 1995–96), arguing against kissing as the dominant route of spread. (Osmond *et al.*, 2002).

The most recent US Public Health Service guidelines for the prevention of KSHV infection reflect the uncertainty in the literature about its transmission (US Public Health Service and Infectious Disease Society of America, 2002). They state that the major routes of KSHV transmission appear to be "oral (via) semen, and through blood via needle sharing" and that patients should be counseled that "kissing and sexual intercourse with persons who have high risk of being infected with KSHV (e.g., persons who have KS or who are HIV–infected), might lead to acquisition of the agent that causes KS." Admittedly, this guideline is rated CIII (meaning evidence for efficacy is insufficient), but it poses a quandary for homosexual men, leaving great uncertainty about whether any form of contact is safe.

In contrast to the data for sexual transmission in homosexual men, evidence for sexual transmission in heterosexual men or women is less convincing. In a large study of 2718 STD clinic attendees in London, KSHV seroprevalence in heterosexual men and women was 4.6% compared to 18.5% in (mostly HIV-uninfected) homosexual men (Smith *et al.*, 1999). Among the heterosexual men and women, there was no association between KSHV seropositivity and either number of sexual partners or current or prior history of STDs. Several other studies have found associations between KSHV seropositivity and STD history (either HIV, genital warts, syphilis, gonorrhea, herpes simplex virus 2, or chlamydial infection) among women (Sosa *et al.*, 1998; Tedeschi *et al.*, 2000; Cannon *et al.*, 2001; Greenblatt *et al.*, 2001; Janier *et al.*, 2002; Goedert *et al.*, 2003), but whether this truly represents sexual transmission rather than a surrogate for other types of incompletely controlled for behavior (e.g., injection drug use) is not clear. Among the two

studies which best controlled for injection drug use by restricting to those women who denied either injection drug use (Cannon *et al.*, 2001) or who were seronegative for hepatitis C virus infection (Goedert *et al.*, 2003), one study reported an association between STD history and KSHV seropositivity (Cannon *et al.*, 2001) but the other did not (Goedert *et al.*, 2003). To date, although one report found a trend towards higher seropositivity in women with earlier sexual debut (de Sanjose *et al.*, 2002), no study has revealed the same unequivocal direct relationship between KSHV seropositivity and number of sexual partners as seen in homosexual men. Moreover, in what should be the most powerful evaluation of heterosexual transmission among women, a history of sex with male bisexual partners has not been associated with KSHV seropositivity (Cannon, *et al.*, 2001; Greenblatt *et al.*, 2001). Given the scarcity of KSHV in semen, the lack of significant heterosexual transmission of KSHV is not surprising. Finally, given the general uncertainty as to whether heterosexual transmission occurs to any appreciable extent in non-endemic areas, there has been no enlightening work as to which specific sexual practices might be operative.

Injection drug use

The paucity of KS among HIV-infected non-homosexual injection drug users (Beral *et al.*, 1990) led to the prediction that the putative agent of KS would be low among injection drug users. Indeed, KSHV seroprevalence in injection drug users is much lower than in homosexual men (Renwick *et al.*, 1998; Simpson *et al.*, 1996) but whether injection drug use can transmit KSHV to any extent is under active investigation. The strongest evidence comes from Cannon *et al.* who evaluated women in the US-based HIV Epidemiology Research Study (HERS) (Cannon *et al.*, 2001). Compared to non-users, injection drug users were more likely to be KSHV-infected in a dose-response manner depending on drug use frequency. Furthermore, when restricted to women with low-risk sexual behavior, those who were hepatitis C virus-infected were 20 times more likely to be KSHV-infected. The inference that KSHV can be transmitted through injection drug use has subsequently been supported in at least 3 other studies in women and men (Diamond *et al.*, 2001; Goedert *et al.*, 2003; Atkinson *et al.*, 2003). In contrast, no association for injection drug use was reported among women in the Women's Interagency HIV Study (WIHS), but this cross-sectional analysis evaluated only the prior 6-month injection drug use history and did not evaluate dose-response trends or an association with HCV seropositivity (a better marker of lifetime injection drug use) (Greenblatt *et al.*, 2001). Similarly, no association was found among injection drug users in Baltimore, but again the study lacked accurate measurement of the lifetime magnitude of injection drug use behavior, critical in a population where everyone has practiced at least some of this behavior (Bernstein *et al.*, 2003). Finally, a study of both male and female drug users in Amsterdam also failed to show an association for injection drug use (Renwick *et al.*, 2002), but admittedly lacked adequate statistical power. Taken together, the available data do support that injection drug use can transmit KSHV. Nonetheless, the efficiency of spread by injection drug use (defined as the probability of transmission per each contact with a KSHV-infected person where injection drug use equipment is shared) is likely very low as evidenced by the markedly lower prevalence in injection drug users as compared to homosexual men. This is consistent with the biological data finding KSHV DNA far more commonly in saliva, a body fluid commonly exchanged during male homosexual activity, than in whole blood, the culprit fluid exchanged among injection drug users.

Blood transfusion

The possibility of transmission by blood transfusion was raised by Blackbourn *et al.* (1997) who recovered KSHV from peripheral blood mononuclear cells from a single blood donor and were able to propagate the virus in previously uninfected target cells. Further concern was introduced with the finding, reviewed above, that injection drug use (i.e., exposure to minute quantities of blood) can apparently transmit KSHV. Confirmation of an actual transmission event in non-endemic areas via blood transfusion, however, has to date not been demonstrated. The most suggestive evidence comes from a study of 284 initially KSHV-seronegative individuals who received a blood transfusion and were followed for 6 months. Two persons developed KSHV seroreactivity by 6 months, compared to no such seroconversions among 75 individuals who did not receive a transfusion (Dollard *et al.*, 2005). If the seroconversions were indeed from the blood transfusion, then the risk of HHV-8 infection per transfused unit was estimated to 0.082% (95% Cl, 0.05 to 0.20%). However, because linked donor specimens were not available and the control group was small in number, it is not possible to definitively prove that the seroconversions were due to the transfusion and not from other sources. Two other smaller look-back studies of a total of 32 known KSHV-antibody-positive blood donors and their associated transfusion recipients failed to detect a seroconversion among the recipients (Operskalasi *et al.*, 1997; Engels *et al.*, 1999). Hence while at this time it cannot be definitively proven or disproven if transmission via blood transfusion occurs in non-endemic settings where modern blood-banking techniques are practiced,

if transmission does occur efficiency is likely to be low. Other epidemiologic data support this view. For transfusion of non-cellular components (e.g., fresh frozen plasma, cryoprecipitate, or factor concentrates), that the risk from transfusion, at least in non-endemic areas, is very low is substantiated by indirect data in (a) hemophiliacs where KSHV seroprevalence is very low (0% to 3%) (Kedes *et al.*, 1996; Gao *et al.*, 1996a,b; Simpson *et al.*, 1996), and (b) HIV-infected hemophiliacs in the 1980's who had a very low incidence of KS. For transfusion of cellular components (e.g., red blood cells), the low incidence of KS among the initial cohort of persons in the 1980s, other than hemophiliacs, who acquired HIV via blood transfusion similarly argues for a very low risk of transmission (Beral *et al.*, 1990). Both the hemophiliacs and red blood cell transfusion recipients were in many cases infected by blood donations from homosexual men who harbored HIV and likely also had a high prevalence of KSHV infection. That so few developed KS strongly suggests that KSHV is very inefficiently spread via transfusion in non-endemic areas with modern blood-banking practices. As such, there has been to date no forceful movement in the US blood banking community to universally screen for KSHV (Dodd 2005). This stance has been further substantiated by the apparent very low seroprevalence of KSHV infection in individuals who have passed the standard medical and behavioral screening for blood donation. In a study of 1000 representative blood donors, KSHV seroprevalence was estimated to be approximately 3% (Pellett *et al.*, 2003). Among the blood donors found to antibody-positive, no donor had detectable evidence of KSHV DNA upon testing of their whole blood.

That KSHV transmission by injection drug use (where only minute quantities of blood are exchanged) has been established in non-endemic areas but transmission by transfusion (where large quantities are exchanged) has not is seemingly inconsistent. The paradox may be explained by the composition of the material exchanged. In injection drug use, fresh whole blood is exchanged, which is replete with mononuclear cells, the host cells for KSHV when it is present in the circulation. In contrast, mononuclear cells are intentionally (albeit not completely) removed in all forms of blood transfusion in most non-endemic areas. Although elements of blood plasma are exchanged in both injection drug use and transfusion, cell-free KSHV DNA is rarely found in plasma. In addition, it has required the presence of multiply exposed injection drug users (e.g., daily drug use over several decades) to delineate a risk associated with this practice. The only comparably exposed group in non-endemic areas to be examined in transfusion medicine were 19 persons from France with thalessemia or sickle cell disease who received a mean of 326 red blood cell transfusions (Lefrere *et al.*, 1997). No instances of KSHV infection were found in these individuals, but, of note, they received only white-blood-cell-reduced (a more complete technique of white blood cell removal than standard processing) red cells, and they were examined for the presence of KSHV only with PCR techniques, not by antibody testing. In a smaller group ($n = 9$) of patients with sickle cell disease who had over 100 red blood cell transfusions and did have KSHV antibody testing performed, two were seropositive, but the sample size was too small to ascertain whether this was significantly greater than the background seroprevalence in the population (Challine *et al.*, 2001).

Organ transplantation

Several studies have confirmed the ability of KSHV to be transmitted through renal transplantation (Regamey *et al.*, 1998; Diociaiuti *et al.*, 2000; Luppi *et al.*, 2000a,b). The initial sentinel work from Switzerland found that 12% (25/206) of renal transplant recipients negative for antibodies to KSHV prior to surgery seroconverted within one year after transplantation, (Regamey *et al.*, 1998). Two of the 25 developed KS. The inference that most, if not all, of these apparent seroconversions were caused by the allograft was strengthened by finding that serum from five out of six donors to seroconverters was positive for KSHV antibodies compared to nought out of eight donors to nonseroconverters. In addition, transmission through blood products was ruled out in several cases. Subsequently, elegant molecular work performed on eight renal transplant recipients with post-transplant KS determined that, among five patients, individual cells microdissected from KS lesions were of donor origin and were KSHV-infected (Barozzi *et al.*, 2003). This finding not only substantiates the ability of KSHV to be transmitted via renal transplantation but implies that, in at least some cases, the virus is spread not as free virus but instead in a cell-associated form in KS progenitor cells. Finally, although the data are not as conclusive, transmission through liver transplantation has also been reported (Andreoni *et al.*, 2001 and Marcelin *et al.*, 2004). Now that transmission through solid organ transplantation has been confirmed, there are beginning to be calls for routine screening of organ donors and recipients (Moore, 2003; Michaels and Jenkins, 2003). Indeed, it is this setting where there is the most urgent need for the development of commercially available high throughput KSHV diagnostic assays. Difficulties in the assays for KSHV antibodies will make this an imperfect process, but the idea seems most warranted in non-endemic areas where the low prevalence of KSHV infection among donors means that few organs will need to be discarded because of KSHV-seropositivity

Table 54.4. Studies evaluating the association between age and KSHV seroprevalence in pre-pubescent children in Africa, the Middle East, and Italy

			Percent KSHV seropositive	
Country/population (reference)	Age group	*N*	Anti-latent Antibody	Anti-lytic Antibody
Cameroon	Age 7–12 mo	32		12.5[a]
Clinic patients	Age 13–24 mo	28		14.3
(Gessain *et al.*, 1999)	Age 3–4	36		13.9
	Age 5–8	34		23.5
	Age 9–11	36		25.0
	Age 12–14	28		39.2
	Age 15–20	27		48.0
	Pregnant women	189		54.5
Uganda	Age 0–5	35	20[b]	28[b]
Hospital outpatients	Age 5–9	48	38	42
(Mayama *et al.*, 1998)	Age 20–24	53	36	46
Egypt	Age <1	42	7.1	16.6
Vaccinees	Age 1–3	40	10.0	37.5
(Andreoni *et al.*, 1999)	Age 4–6	40	7.5	45.0
	Age 7–9	38	10.5	57.8
	Age 10–12	36	5.5	52.7
Italy	Age 3–5	90	1.1	
Hospital inpatients	Age 6–10	94	6.3	
(Whitby *et al.*, 2000)	Age 11–15	38	7.8	

[a] an immunofluorescence assay detecting antibodies to both lytic and latent-phase antigens was used.
[b] percentages extrapolated from figure.

and those that are may be able to be given to recipients who are seropositive.

Transmission in endemic areas

Non-sexual horizontal transmission

In high-level endemic areas, such as the Middle East and Africa, an age-dependent increase in KSHV seroprevalence during childhood provides definitve evidence for some form of non-sexual horizontal transmission (Table 54.4). In intermediate-level endemic areas, e.g., the Mediterranean, fewer comparable data exist (Perna *et al.*, 2000; Whitby *et al.*, 2000); although only one report is suggestive of an age-dependent increase in KSHV seroprevalence before puberty (Whitby *et al.*, 2000), this is substantiated by the finding of increased seroprevalence in the children of patients with KS, relative to persons without KS (Angeloni *et al.*, 1998). Indeed, that prevalence at the time of puberty in some high-level endemic areas is nearly that seen in adulthood suggests that non-sexual horizontal transmission is the dominant route of spread; (this, of course, assumes that seroreversion is not common and therefore not masking the occurrence of a continuing high incidence of infection in adulthood). For example, in Cameroon, Gessain *et al.* demonstrated that after loss of maternal antibody by 6 months of age, there was a monotonic increase in KSHV seroprevalence from 13% in 7- to 24- month olds to 39% in 12- to 14-year-olds (Gessain *et al.*, 1999). There was a similar pattern found among Ugandan children (Mayama *et al.*, 1998). In Egypt, prevalence was 17% in 0- to 1-year-olds ranging to 58% among 7 to 9 years (Andreoni *et al.*, 1999).

That saliva is the body fluid that most commonly harbors KSHV lends the biologic plausibility that some form of non-sexual horizontal transmission is the dominant form of spread in endemic areas. The exact mode of spread, however, is not known. Some of the first reports from sub-Saharan Africa found that maternal KSHV seropositivity was a risk factor for seropositivity among children, leading to the conclusion that mother-to-child spread was the most significant route (Bourboulia *et al.*, 1998; Sitas *et al.*, 1999a,b). These data, however, were not controlled for the family's community of residence (and hence

background prevalence of KSHV infection), and did not consider whether this association might be confounded by father-to-child, sibling-to-sibling, or extrafamilial spread. In a cross-sectional study that evaluated all family members, Plancoulaine *et al.* (2000) found evidence for both mother-to-child and sib-to-sib spread, but not father-to-child or spouse-to-spouse. This, however, was performed in French Guiana, where a seroprevalence of only 15% among persons 15 to 40 years old was reported, even when using a more sensitive induced IFA antibody assay. Whether or not the findings apply to areas with much higher prevalence, such as Africa, is not clear. A similar cross-sectional family study from Israel, where again seroprevalence among adults was only slightly greater than 10%, found a role for mother-to-child spread but did not examine the role of sib-to-sib spread (Davidovici *et al.*, 2001). Most recently, a study of children in a high prevalence area in rural Tanzania found that having a KSHV-seropositive mother, father, or next-older sibling was associated with being KSHV-seropositive and that the magnitude of the risk was about the same across these relatives (Mbulaiteye *et al.*, 2003a,b). Importantly, there was also an age-dependent increase in KSHV seroprevalence among children who did not have a seropositive first-degree relative, suggesting that non-familial transmission also occurs. Transmission emanating from outside of the immediate family has also been reported in initial molecular epidemiologic work performed among family members in Malawi (Cook *et al.*, 2002a, b).

Taken together, these studies suggest that a variety of relationship types (i.e., mother–child, father–child, sib–child, non-family member–child) may be important in KSHV transmission. It is likely that no single relationship type has any universal significance per se in transmitting KSHV but rather that transmission is a product of salivary shedding in the infected individual and as-of-yet undetermined sociocultural practices that promote saliva passage to either interrupted skin or mucosal surfaces in the uninfected child. Some interesting theories have been proposed but for which supportive data are currently lacking. One of the most speculative put forward is the blood-sucking promoter arthropod hypothesis which suggests that the bite of a child by a bloodsucking arthropod triggers two subsequent events: (a) transfer of anti-hemostatic (anticlotting, vasodilators, and anti-platelet agents) and immunodulators from the arthropod to the human skin; and (b) use of saliva by a caregiver to relieave the itch of the bite (Coluzzi *et al.*, 2002, 2003, 2004 and Ascoli *et al.*, 2006). In this theory, the arthropod is not the biological vector for KSHV, but rather a promoter of infection by creating a hospitable microenvironment in the skin for KSHV infection and then facilitating contact with KSHV-containing saliva. As has been the case for horizontal transmission of hepatitis B virus infection in endemic areas, identifying the precise mechanisms by which KSHV is spread to children will be a considerable challenge. The exact mode of spread notwithstanding, why non-sexual horizontal transmission in children is so common in one part of the world but so uncommon in others (e.g., the USA) is not understood. While an association between lower education and KSHV seropositivity in some studies in endemic areas (Sitas *et al.*, 1999a,b; Newton *et al.*, 2003) suggests that region-specific behavioral factors may be important, the role of region-specific differences in host-meditated response to KSHV remains largely unexplored. Although a culprit gene has not yet been identified, segregation analysis in a French Guiana population suggests that a recessive gene controlling susceptibility to KSHV infection may explain the rapidly rising KSHV seroprevalence up to puberty in the population with a subsequent plateau thereafter (Plancoulain *et al.*, 2003).

Sexual transmission

While sexual transmission among homosexual men appears to occur in KSHV-endemic areas just as it does in non-endemic areas (Perna *et al.*, 2000; Rezza *et al.*, 1998), data on heterosexual transmission in endemic areas are conflicting. That sexual transmission may occur is evidenced in some areas where there is a monotonic increase in KSHV seropositivity through adulthood (Olsen *et al.*, 1998; Sitas *et al.*, 1999a,b; Newton *et al.*, 2003; Lavreys *et al.*, 2003) or where risk of KSHV infection among married individuals is associated with KSHV-seropositivity or presence of KS *per se* in their spouses (Mbulaiteye *et al.*, 2003a,b; Brambilla *et al.*, 2000). Alternatively, these finding might simply be explained by continued non-sexual transmission during adulthood. The best evidence for heterosexual transmission in intermediate-level endemic areas comes from one report from Sicily showing an association between prostitution, STD clinic attendance and KSHV-seropositivity (Perna *et al.*, 2000), although others have not confirmed this (Masini *et al.*, 2000). In high-level endemic areas, the best evidence is from two studies from Cameroon (Bestetti *et al.*, 1998, Rezza *et al.*, 2000a,b), two from Kenya (Lavreys *et al.*, 2003; Baeten *et al.*, 2002), and one from Nigeria (Eltom *et al.*, 2002a,b) each showing a relationship between KSHV-seropositivity and prostitution and/or history of at least one STD. The work from Kenya was also notable for associations between KSHV seropositivity and lack of condom use and lack of circumcision. (Baeten *et al.*, 2002). However, several other studies have found no role for heterosexual transmission (Mayama *et al.*, 1998; Newton *et al.*, 2003; Rezza *et al.*, 1998; Enbom *et al.*, 1999; Wawer

et al., 2001; Vitale *et al.*, 2000; Marcelin *et al.*, 2002; Enbom *et al.*, 2002). In particular, there is marked inconsistency in high-level endemic areas between what should be a powerful marker of sexual behavior – HIV infection – and KSHV serostatus with some studies showing an association (Bestetti *et al.*, 1998; Nuvor *et al.*, 2001; Hladik *et al.*, 2003) but others not (Sitas *et al.*, 1999; Rezza *et al.*, 2000; Baeten *et al.*, 2002; Marcelin *et al.*, 2002; He *et al.*, 1998). This is in contrast to the universal association seen between HIV infection and KSHV serostatus in homosexual men in low-level endemic areas (Martin *et al.*, 1998; Renwick *et al.*, 1998; O'Brien *et al.*, 1999). In terms of direct information on number of sexual partners in endemic areas, Sitas *et al.* (1999a,b) found a very small risk associated with lifetime sexual partners but others have not confirmed this (Lavreys *et al.*, 2003; Baeten *et al.*, 2002). Of particular acts examined, the one report that examined kissing did not show an association with KSHV seropositivity (Lavreys *et al.*, 2003).

To date, no longitudinal studies evaluating sexual transmission of KSHV that follow initially seronegative adults have been performed. Given the high prevalence of KSHV infection that is already present by puberty in endemic areas, attempts with cross-sectional studies to assess the role of either current or past sexual behavior in determining KSHV serostatus are methodologically challenging. The expectation of these cross-sectional studies would be associations that are attenuated. Therefore, definitive evidence will likely await longitudinal study.

Vertical transmission

The occurrence of KS in young children, and, in particular, a case report of KS in a 6-day-old child (Gutierrez-Ortega *et al.*, 1989) has suggested that KSHV can be vertically transmitted. Because of the presence of maternal antibody in infants, documentation of vertical transmission relies upon detection of KSHV DNA. Mantina *et al.* (2001) evaluated the newborns of 89 Zambian women who were KSHV-antibody positive. In a blood draw taken at 24 hours of life, 2 of 89 (2.2%) had detectable KSHV DNA in peripheral blood cells by polymerase chain reaction (PCR). Because KSHV DNA sequences could not be detected from the mothers, it cannot be determined whether or not these two cases represent true vertical infection or PCR contamination. Even if they do represent true infection, it would appear that the incidence and efficiency of vertical infection is quite low.

Blood transfusion

Because of the high prevalence of KSHV infection in endemic areas, if transmission via blood transfusion occurred, even if inefficient, it could pose a significant public health concern. A study of Ugandan children with sickle-cell disease found a dose–response relationship between number of transfusions received and KSHV-seropositivity, even after adjustment for age (Mbulaiteye *et al.*, 2003). The overall estimated KSHV transmission risk was 2.6% (95% confidence interval 1.9% to 3.3%) per unit transfusion. If it is assumed that the prevalence of KSHV among donors in Uganda is approximately 50%, then the risk associated with receipt of a transfusion from a KSHV-seropositive donor is 5%. This finding of KSHV transmission via blood transfusion was recently confirmed in a longitudinal study, also conducted in Uganda (Hladik *et al.*, 2006). This work also showed that blood units stored less than 4 days prior to use were associated with a higher risk of KSHV transmission than units stored for more than 4 days. Of note, however, is that in Uganda transfusion recipients are typically given fresh unprocessed whole blood. Whether or not these same per unit risks occur when fractionated blood products (e.g., leukocyte-depleted red blood cells or plasma) are used, as is the case in more developed settings, is not known. The finding of KSHV DNA in the serum of a substantial proportion of blood donors in at least one African population lends credence to the (Enbom *et al.*, 2002) concern.

Disease manifestations

Primary infection syndrome

Very little is known about the clinical manifestations of primary KSHV infection. A major reason for this is the absence of clinically available diagnostic tests that could be used to evaluate large numbers of symptomatic persons. Furthermore, the phenomenon of seroreversion, which occurs with the currently available research-level serologic assays, makes identification of seroconversion uncertain in the absence of long-standing and repeated documentation of seronegativity (as would only be available in a formal cohort study). As such, in adults, all that is known comes from three reports of what are believed to represent primary infection. In the first, an HIV-infected man developed a sudden but transient onset of angiolymphoid hyperplasia with fever, arthralgia, cervical adenopathy, and splenomegaly (Oksenhendler *et al.*, 1998). The second report involved two transplant recipients who each received a kidney from same KSHV-seropositive donor; one recipient developed disseminated KS and the other had fever, splenomegaly, and pancytopenia (Luppi *et al.*, 2000a,b). These reports emphasize the potential severity of primary infection, but because they were identified on the basis of symptoms

they do not indicate what percentage of new infections are associated with clinical symptoms. Moreover, the reports may not represent primary infection in immunocompetent hosts. The third report described five cases of KSHV seroconversion that developed during the longitudinal observation of HIV-uninfected homosexual men (Wang *et al.*, 2001). Four of the five had one or more symptoms of lymphadenopathy, diarrhea, fatigue, or rash. Although the symptoms were self-limiting, their longevity and severity were difficult to ascertain from the retrospective study design. Furthermore, this study reported a KSHV incidence of only 3.7 cases/1000 person–year, which seemingly could not account for the prevalence of 9.2% observed at the beginning of the cohort's observation, raising questions about whether the seroconversions detected represented only the cases that were the most overt immunologically and perhaps clinically. Nonetheless, this 4:1 ratio of symptomatic to asymptomatic infection is intriguing and merits further dedicated prospective study.

In children, a study of 86 one- to four-year olds in Egypt with fever of undetermined origin found six children with KSHV DNA in saliva but without KSHV antibodies, thus presumably representing primary infection (Andreoni *et al.*, 2002). Of the three children for whom a follow-up blood sample was obtained, all developed KSHV antibodies. In addition to fever, five of the six children developed a rash that first appeared on the face and gradually spread to the trunk, arms, and legs. Five of the six also had upper respiratory tract infection symptoms and two had lower respiratory tract infection symptoms. While this report again demonstrates the severity of disease that can occur, its hospital-based sampling precludes an understanding of the overall frequency of symptomatic disease among children with primary infection. There has also been one report of a 1-month-old girl with DiGeorge anomaly, a primary immunodeficiency, who had widespread KSHV dissemination with virus detected in peripheral blood mononuclear cells, bone marrow, spleen, and lymph nodes as well as in endothelial and epithelial cells of the skin, lungs, esophagus, intestine, choroid plexus, and heart (Sanchez-Velasco *et al.*, 2001). She died of multi-organ failure. Of interest from a transmission viewpoint is that there was no evidence of KSHV infection in her mother.

Kaposi's sarcoma

There are four forms of KS, defined on the basis of their epidemiologic context. "Classic" KS is the syndrome originally described by Kaposi (1872), and it has recently been thoroughly reviewed (Iscovich *et al.*, 2002). It is found most frequently in older men (over 50 years) of Mediterranean or Eastern European heritage and is characterized initially by bluish-red painless spots on the feet and lower legs that subsequently progress, if untreated, to form raised, nodular growths that may ulcerate and bleed. In the latter stages of disease, the entire lower extremities as well as the hands and arms may become involved. Although the disease course is typically indolent, internal involvement does occur in approximately 10% of cases.

African or "endemic" KS is most common in sub-Saharan Africa. Even before the AIDS epidemic, portions of sub world Saharan Africa, particularly the Nile-Congo watershed, had among the highest incidences of KS in the world (Davies *et al.*,1964; Hutt and Burkitt, 1965). In these areas, KS represented 4 to 10% of all adult cancers and was mainly a disease of men aged 30 to 50 years old. Like classic KS, endemic KS manifests primarily as localized plaques and nodules on the feet and lower legs, but visceral involvement is more common than in the classic form.

The third form of KS was described shortly after the advent of solid organ transplantation (Siegel *et al.*, 1969; Penn, 1979), and foreshadowed the powerful influence of immunosuppression on KS development. In this form, called "post-transplant" or "iatrogenic" KS, both disseminated cutaneous involvement and visceral disease are common. As noted earlier, recent work has shown that among cases that develop because of de novo transmission of KSHV, cells within the KS lesions are often of donor origin (Barozzi *et al.*, 2003). This implies that either circulating KSHV-infected spindle-like cells or lymphoid cells trapped in the allograft or endothelial cells (or their precursors) residing in the allograft are the critical transplanted element that leads to KS lesion formation. Interestingly, lesions often diminish with lessening of the immunosuppressive regimen (Penn, 1979), but because this reduction is often accompanied by organ rejection, KS is a feared post-transplant complication. The finding that KS lesions are sometimes of donor cellular origin implies that donor-derived KSHV-specific cytotoxic T-cells could be a useful form of therapy that could obviate the need to lessen immunosuppression.

The fourth form of KS, originally described in young homosexual men, heralded the beginning of the HIV epidemic in 1981 (Fig. 54.2) (Friedman-Kien, 1981; Hymes *et al.*, 1981). "Epidemic" or "AIDS-associated" KS became the most common HIV-associated malignancy, particularly affecting persons who acquired HIV via male homosexual behavior (Beral *et al.*, 1990). Prior to the introduction of effective anti-HIV therapy, HIV-infected homosexual men were estimated to have a 20-fold higher risk of KS development than other HIV transmission risk groups (Beral *et al.*, 1990) a lifetime cumulative incidence of

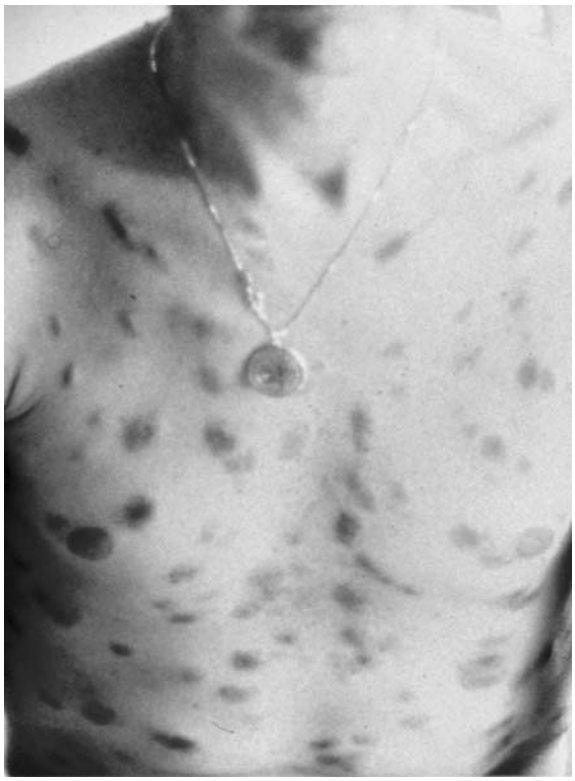

Fig. 54.2. Diffuse cutaneous Kaposi's sarcoma in a homosexual man.

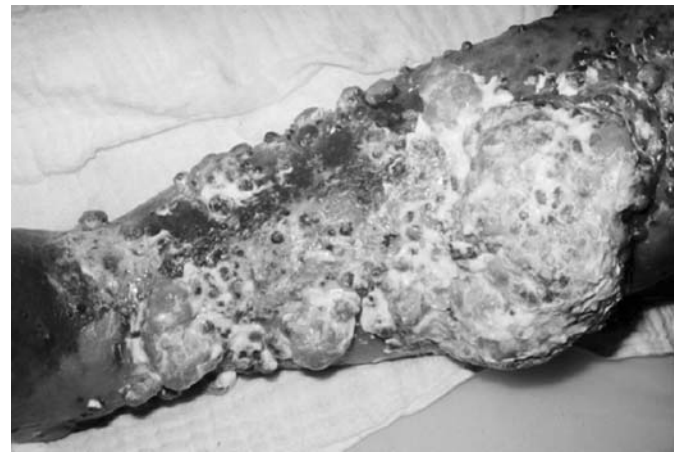

Fig. 54.3. Fulminant nodular Kaposi's sarcoma of the lower extremity in a patient with AIDS.

37% (Hoover *et al.*, 1993). Cutaneous AIDS-related KS, typically the earliest presentation, is often disseminated, cosmetically disfiguring, and complicated by bulky lesions, lymphatic obstruction, and extremity or facial swelling (Fig. 54.3) (Kaplan and Northfelt, 1997). Subsequent visceral manifestations, especially pulmonary and gastrointestinal involvement, are common (Friedman *et al.*, 1985; Mitchell *et al.*, 1992) and convey a poor prognosis. Prior to 1996 (i.e., prior to the era of highly active antiretroviral therapy for HIV and improved chemotherapy for KS), the median survival for HIV-infected persons with visceral involvement was 15 months (Krown *et al.*, 1997). However, even persons with KS confined to the skin and/or lymph nodes also had significant mortality with a median survival of 27 months (Krown *et al.*, 1997). As it has with other complications of HIV infection, the advent of highly active antiretroviral therapy has dramatically altered the prognosis of AIDS-associated KS. In a study of 287 patients with KS from 1990–1999, use of highly active antiretroviral therapy was associated with an 81% reduction in mortality (Tam *et al.*, 2002).

The introduction of potent antiretroviral therapy for HIV infection in the mid-1990s has also led to a dramatic reduction in the incidence of AIDS-associated KS in resource-rich settings (Eltom *et al.*, 2002a,b; Portsmouth *et al.*, 2003). In contrast, in portions of sub-Saharan Africa, where KSHV is endemic and antiretroviral therapy is not widely available, KS is now the most common adult malignancy. For example, in Uganda the incidence of KS has increased more than 20-fold in men between 1964 and 1968 and 1989 and 1991 (Wabinga *et al.*, 1993). A similar situation exists in Zimbabwe, where the HIV epidemic is largely unchecked and where KS is now the most common cancer overall in adults, most common in men (40% of all cancers) and the second most common in women (18% of all cancers) and children (10% of all cancers) (Chokunonga *et al.*, 2000). As is seen in AIDS-associated KS in resource-rich settings, the clinical manifestations of KS in sub-Saharan Africa have also changed. The most common presentations are now more aggressive forms such as lymphadenopathic or visceral KS, for which effective, affordable treatment is generally unavailable (Wabinga *et al.*, 1993; Desmond-Hellmann *et al.*, 1991).

The disproportionate risk of KS among HIV-infected homosexual men led to the hypothesis that a sexually transmitted infectious agent, other than HIV, was etiologically important (Beral *et al.*, 1990). This epidemiologically driven hypothesis re-invigorated an intense laboratory investigation for the causative agent that ultimately resulted in the identification of KSHV (Chang *et al.*, 1994). In a relatively short period of time since the discovery of KSHV, there have been a large number of epidemiologic studies evaluating its causal role in KS (Table 54.5). In the absence of experimental evidence (e.g., Koch's postulates), epidemiologists assess causality using Hill's criteria (Hill, 1965). These

Table 54.5. Studies estimating the association between KSHV infection and AIDS-related Kaposi's sarcoma[a]

Reference	KSHV antigen targeted	Antibody assay format	Association between KSHV and AIDS-KS, point estimate (95% CI)[b]
Case-control studies			
Miller *et al.*, 1996	p40	immunoblot	13.4 (4.5–42)
Miller *et al.*, 1996	lytic cycle	IFA[d]	12.2 (4.1–38)
Gao *et al.*, 1996a,b	LNA[c]	immunoblot	18.9 (5.4–70)
Kedes *et al.*, 1996	LNA	IFA	8.5 (3.1–23)
Gao *et al.*, 1996a,b	LNA	IFA	16.3 (5.3–50)
Simpson *et al.*, 1996	LNA	IFA	10.2 (4.2–25)
Simpson *et al.*, 1996	ORF65	EIA[e]	9.2 (2.7–31)
Lennette *et al.*, 1996	lytic cycle	IFA	1.8 (0.53–5.8)
Lennette *et al.*, 1996	LNA	IFA	4.2 (2.2–8.0)
Longitudinal studies			
Martin *et al.*, 1998	LNA	IFA	2.5 (1.7–3.8)
Renwick *et al.*, 1998[f]	ORF65.2 ORF73	EIA	3.3 (1.9–5.8)
Renwick *et al.*, 1998[g]	ORF65.2 ORF73	EIA	5.2 (2.9–9.3)
O'Brien *et al.*, 1999	LNA	IFA	3.6 (1.7–9.5)

[a]limited to studies which measure KSHV infection by antibody response and either directly control for number of sexual partners or restrict analyses to HIV-infected homosexual men
[b]odds ratios are reported for case-control studies and hazard ratios for longitudinal studies
[c]latent nuclear antigen
[d]immunofluorescence assay
[e]enzyme immunoassay
[f]hazard ratio shown is for KSHV prevalent infection
[g]hazard ratio shown is for KSHV seroconverters.

criteria are discussed in Table 54.6 in order of their importance. The overwhelming assessment of these criteria, coupled with the finding that KSHV is invariably present in KS tumor specimens from all forms of KS throughout the world, is that KSHV is a necessary causal agent of KS. However, because only a small fraction of persons infected with KSHV develop KS, it is concluded that KSHV is a necessary, but not sufficient, causal agent of KS.

Of other potential cofactors operating along with KSHV in the etiology of KS, immunosuppression in general, and HIV infection, in particular, confer the greatest risk. Among HIV-infected persons, the magnitude of immunosuppression, as evidenced by CD4+ T-cell count, is predictive of development of KS (Renwick *et al.*, 1998; Jacobson *et al.*, 2000a,b). Timing of KSHV infection relative to HIV infection may be important as well as the risk of developing KS was significantly greater in one study (Renwick *et al.*, 1998), and of similar magnitude but not statistically significant in a second study (Jacobson *et al.*, 2000a,b), in persons who acquire KSHV after as compared to before HIV infection. Studies of this nature have potential methodologic pitfalls (Cannon and Pellett, 2001), however, and could conceivably be explained by what appears to be KSHV seroconversion actually being KSHV reactivation and reappearance of antibody-positivity. Two potential genetic risk factors have also been described and await confirmation. The FF genotype of the low affinity Fc gamma receptor IIIA is underrepresented in patients with KS, whereas the VF genotype confers greater risk (Lehrnbecher *et al.*, 2000). Homozygosity for allele G at amino acid 174 of the IL6 promoter, associated with increased IL6 production, has been related to the development of KS while homozygosity of allele C appears protective (Foster *et al.*, 2000). However, it is not known whether these genetic factors influence development of KS via pathogenic acceleration among KSHV-infected persons or via enhancing the acquisition of KSHV *per se*.

Despite substantial investigation, the identification of cofactors, in addition to KSHV, for development of KS among immunocompetent individuals is largely incomplete. The role of cofactors is particularly evident in Africa, where prior to the AIDS epidemic, there was tremendous heterogeneity in KS incidence; this heterogeneity is not fully explained by underlying geographic differences in KSHV prevalence (Dedicoat and Newton, 2003). The absence of KS among Ethiopian immigrants to Israel compared to Israeli-born residents, despite higher KSHV

Table 54.6. Assessment of the Hill criteria for the role of KSHV infection in the etiology of Kaposi's sarcoma

Criterion	Comment
Temporality	Demonstrating that KSHV infection precedes KS is the *sine qua non* for causality. KSHV infection has been shown to precede KS in several studies (Whitby *et al.*, 1995; Gao *et al.*, 1996; Martin *et al.*, 1998; Renwick *et al.*, 1998; O'Brien *et al.*, 1999; Moore *et al.*, 1996; Lefrere *et al.*, 1996) thus dispelling notions that KS tumors, by being hospitable breeding grounds, result in the subsequent presence of KSHV. Importantly, KSHV infection is temporally associated with KS independent of degree of sexual exposure (Martin *et al.*, 1998) (excluding the possibility that KSHV is just a marker for another as-yet-undiscovered sexually transmitted pathogen) and immunocompromise (Whitby *et al.*, 1995; Gao *et al.*, 1996; Martin *et al.*, 1998; Renwick *et al.*, 1998; O'Brien *et al.*, 1999).
Strength of Association	After adjusting for all known potential confounding factors, statistical associations between a putative cause and disease that maintain a large magnitude are less likely to be explained by unaccounted for confounding factors and hence more likely to be valid associations. In longitudinal studies of KSHV and KS, the relevant association to be evaluated is KS incidence in KSHV-infected persons compared to KSHV-uninfected persons. If it is accepted that KS *per se* does not cause KSHV infection to be detected, associations from case-control studies are also relevant. Of studies of AIDS-related KS that measured KSHV infection by serologic means and directly controlled for number of sexual partners (Martin *et al.*, 1998) or restricted analyses to homosexual men (Gao *et al.*, 1996; Renwick *et al.*, 1998; O'Brien *et al.*, 1999; Miller *et al.*, 1996; Kedes *et al.*, 1996; Gao *et al.*, 1996; Simpson *et al.*, 1996; Lennette *et al.*, 2002) the magnitude of association between KSHV and KS ranges from 2.5 to 5.2 (hazard ratios) in longitudinal studies and 1.8 to 18.9 (odds ratios) in case-control studies.
Consistency	All published epidemiologic reports addressing a possible association between KSHV and, KS, with the exception of one have found a direct association. While it is formally possible that an unrecognized bias in either subject selection or variable measurement could account for the association between KSHV and KS seen in all studies to date, this is improbable because such a bias is unlikely to be consistently present in such a wide array of studies which vary by design, participant selection, and assay formats. Furthermore, the remarkable consistency in results makes chance a very unlikely explanation.
Biologic Plausibility	There are several potential mechanisms by which KSHV may cause, KS, described in Chapter 56.
Dose-Response Relationship	Among HIV and KSHV co-infected homosexual men, this criterion has been satisfied by the finding that detectable peripheral blood mononuclear cell-associated KSHV is associated with the subsequent development of KS (Engels *et al.*, 2003).
Coherence	This criterion implies that a cause-and-effect interpretation for the association in question does not conflict with what is otherwise known about either the disease or the putative causal agent. A causal role for KSHV in KS is compatible with existing knowledge in that the epidemiology of KSHV *per se* matches that long predicted for the agent of KS (e.g., highest prevalences in groups at greatest risk for, KS, and, in developed settings, sexually transmitted).
Analogy	Although a weak criterion, there is analogous evidence for a causal role of KSHV in KS. Two herpesviruses closely related to KSHV, herpesvirus saimiri and Epstein-Barr virus (EBV), are oncogenic.
Specificity	That a single cause must cause a single disease is clearly outdated (Rothman and Greenland, 1998) and likely violated by KSHV which has also at least been associated with primary effusion lymphoma (Cesarman *et al.*, 1995) and multicentric Castleman's disease (Soulier *et al.*, 1995).
Experimental evidence	In SCID mice, injection of KSHV into transplanted human skin results in the formation of lesions that are morphologically and phenotypically consistent with KS (Foreman *et al.*, 2001).

seroprevalence in the Ethiopians, is a well-documented example of some heretofore undiscovered protective factor (Grossman *et al.*, 2002). Studies which have evaluated factors associated with KS that were performed prior to the identification of KSHV have had their interpretations clouded by the possibility that these factors are associated with acquisition of KSHV. This pertains to, for example, genetic studies where in at least some reports an increased risk for classic KS has been observed in persons with HLA DR5 and a protective effect observed for HLA DR3 (Contu *et al.*, 1984). Both male gender and older age have for long been identified as a significant risk factors for KS, and neither can be fully explained by an increased risk for acquisition of KSHV infection. However, mechanistic explanation

Table 54.7. Current strength of evidence* for a causal role of KSHV infection in various diseases

Disease	Strength of evidence
Kaposi's sarcoma	+ + +
Primary effusion lymphoma	+ +
Multicentric Castleman's disease	+ +
Hemophagocytic lymphohistiocytosis	+ +
Pulmonary hypertension	+
Prostate cancer	+
Amyotrophic lateral sclerosis	+
Multiple myeloma	−
Sarcoidosis	−

+ denotes weak evidence; + + denotes moderate evidence;
*+ + + denotes strong evidence;
− denotes that initial claims have been largely refuted.

for these factors is unknown. Higher levels of immune activation, measured by circulating neopterin and beta-2-microglobulin, have also been associated with classical KS, but it has not been determined whether this is a cause or a consequence of KS (Touloumi *et al.*, 1999). More recent studies that were either limited to participants with KSHV infection or adjusted for KSHV infection are most notable for the absence of demonstrable associations. Nonetheless, two new potential behavioral factors have been identified that require further investigation. In classic KS, smoking was found to be protective; the effect of smoking on inflammatory cytokines was the speculated mechanism (Goedert *et al.*, 2002). In endemic KS, going barefoot more than half the time was a risk factor (Ziegler *et al.*, 2003). It has been suggested that fine soil particles might pass through the skin of barefoot individuals and block the lymphatic system, causing local immunosuppression in the lower limbs and predisposing to the development of KS (Ziegler, 1993).

Other diseases

KSHV also has been associated with primary effusion lymphoma (Cesarman *et al.*, 1995), multicentric Castleman's disease (Soulier *et al.*, 1995), and hemophagocytic lymphohistiocytosis (Fardet *et al.*, 2001) (Table 54.7). The rarity of these disorders will likely preclude their ever being scrutinized with Hill's criteria, as was discussed for KS. Among other diseases that have been hypothesized to be caused by KSHV, multiple myeloma (Rettig *et al.*, 1997) has generated the most interest because of its frequency andclinical severity. From an ecological viewpoint, the association lacks plausibility given that multiple myeloma is not more common in populations in which KSHV prevalence is high. In addition, numerous individual subject-level studies (Parravicini *et al.*, 1997; Masood *et al.*, 1997; Whitby *et al.*, 1997; Ablashi *et al.*, 2000; Brander *et al.*, 2002) have failed to confirm the initial claim. Most recently, claims have also been made for an association between KSHV infection and pulmonary hypertension (Cool *et al.*, 2003), prostate cancer (Hoffman *et al.*, 2004), and amyotrophic lateral sclerosis (Cermelli *et al.*, 2003). The clinical impact of these conditions, especially prostate cancer, is sure to prompt much additional work in the next several years seeking to confirm these associations.

Acknowledgments

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EBV-induced oncogenesis

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Introduction

The Epstein–Barr virus (EBV) is a human herpesvirus that is a ubiquitous infectious agent, infecting greater than 90% of the world's population (Henle *et al.*, 1969). The majority of infections occur early in life without significant illness. However, EBV is clearly an important factor in multiple human cancers. This dichotomy raises the question as to what are the unique aspects of infection in those who develop cancers. The study of EBV and its associated tumors points to specific interactions between environmental, genetic, and viral factors.

Many of the malignancies associated with EBV develop in the setting of immunosuppression or have endemic patterns of incidence (Raab-Traub, 1996). EBV has potent growth transforming properties in vitro where it efficiently induces permanent growth of infected lymphocytes (Nilsson *et al.*, 1971). Therefore it is not surprising that EBV is clearly the etiologic factor in post-transplant lymphoma and a subset of AIDS-associated lymphomas.

The endemic patterns of incidence characteristic of many EBV-associated tumors were initially apparent as EBV was originally isolated from samples of African Burkitt's Lymphoma (BL) (Epstein *et al.*, 1964). This childhood tumor develops with high incidence in subequatorial Africa (Burkitt, 1962a). EBV was subsequently identified in nasopharyngeal carcinoma (NPC), a major tumor that occurs with extraordinarily high incidence in the southern Chinese and with elevated incidence in Inuit populations and in Mediterranean Africa (Henle *et al.*, 1978a; Wolf *et al.*, 1973). EBV has also been identified in a subset of T-cell lymphomas that develop with increased frequency in Taiwan and Japan and in parotid gland tumors that occur most frequently among Inuits (Su *et al.*, 1990; Saemundsen *et al.*, 1982).

Finally, there are the more recently identified tumors associated with EBV, a subset of Hodgkin's lymphoma (HL) and gastric carcinoma (Weiss *et al.*, 1989a,b; Shibata *et al.*, 1991). The factors that contribute to the development of these tumors are unknown but the environmental and immune components may be similar to those that contribute to the development of EBV cancers with endemic incidence patterns or due to immune impairment. It is likely that environmental or genetic factors increase infection of distinct susceptible cell populations and activate cellular pathways that are highly synergistic with EBV genes that affect cell growth.

This chapter will review the pathogenesis of EBV infection, the characteristics of EBV-associated tumors, the molecular biology of latent infection, and biochemical properties of EBV genes as they relate to tumor development.

EBV infection in vivo

EBV is the prototype member of the Herpesvirus subfamily, Gammaherpesviridae, and like other herpesviruses, the virus establishes a latent infection with life-long persistence in the infected host (Kieff and Rickinson, 2001). The virus is transmitted by salivary exchange and may initially infect the epithelial cells of the oropharynx, posterior nasopharynx, parotid gland and duct, and possibly tonsilar lymphocytes (Miller *et al.*, 1973; Wolf *et al.*, 1984; Sixbey *et al.*, 1984). Hybridization in situ has detected evidence of EBV DNA in cells lining the parotid duct and EBV DNA and replicative mRNAs have been detected in sloughed oropharyngeal epithelial cells and tonsilar lymphoid cells (Sixbey *et al.*, 1984). Through infection of trafficking lymphocytes, the virus then establishes a

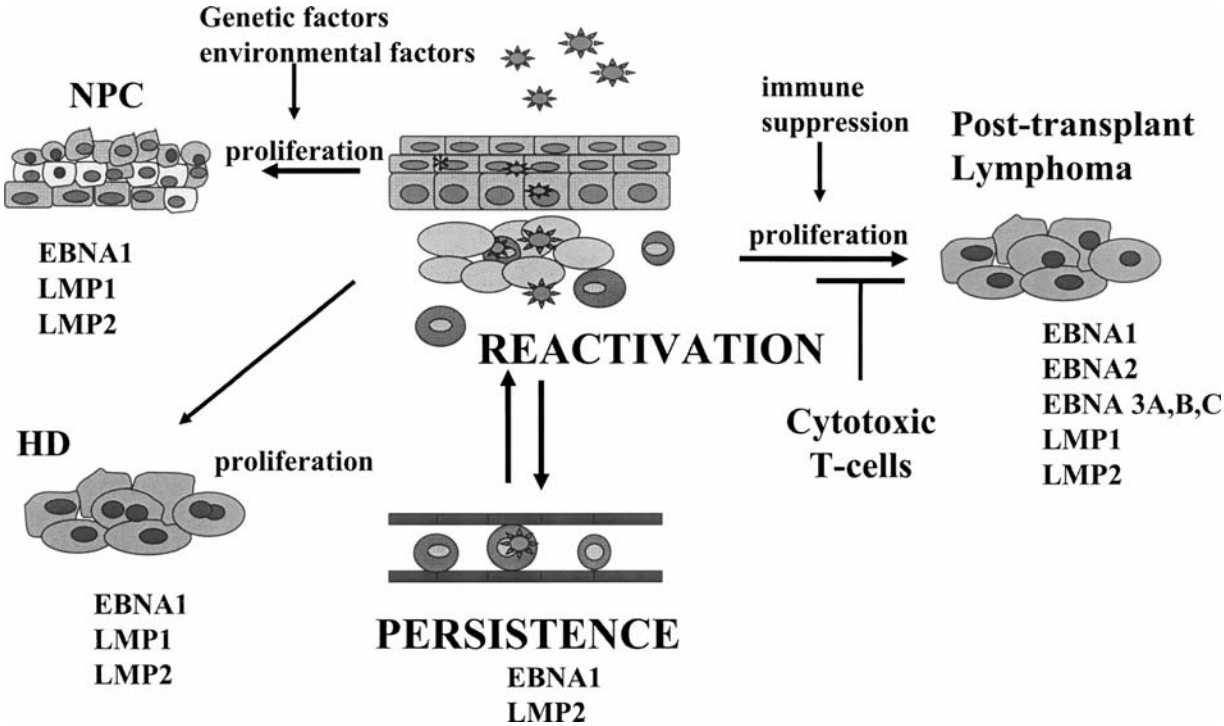

Fig. 55.1. EBV pathogenesis. EBV is transmitted by saliva and infects oropharyngeal epithelial cells and lymphocytes. The virus persists in memory B cells that have limited EBV expression, possibly only EBNA1 or LMP2. The virus frequently reactivates from peripheral blood lymphocytes producing virus and may be directly shed from lymphocytes into the nasopharynx or may replicate in mucosal epithelial cells. In combination with possible genetic changes such as the absence of p16 expression, the virus may establish a latent infection in a basal epithelial cell. Additional genetic changes may enable expression of EBNA1, LMP1, and LMP2 which may not be presented by specific HLA types. The viral episome is maintained in the infected epithelial cell which rapidly becomes malignant in nasopharyngeal carcinoma (NPC). Similar events may occur in the development of Hodgkin Disease (HD). In the context of immunosuppression, the virus can reactivate to full latent expression with expression of EBNA2, 2, 3A,3B,3C, LMP1, and LMP2. The transformed cells may develop into post-transplant lymphoma in the absence of cytotoxic T-lymphocytes.

persistent latent infection in bone marrow and peripheral blood memory B-lymphocytes (Fig. 55.1). Virus expression is tightly restricted in latently infected B-lymphocytes with no viral gene expression or with expression limited to the EBV nuclear antigen 1 (EBNA1), and/or latent membrane protein 2 (LMP2) (Babcock *et al.*, 2000a). The state of EBV latency may reactivate and express additional viral genes that induce cell growth, including the other EBNAs and LMP1 and LMP2. The proliferation of the infected lymphocytes is controlled by cytotoxic T-cells (CTLs) that primarily recognize the EBNA2 and EBNA3 proteins (Murray *et al.*, 1990). Therefore in immunosuppressed patients who lack CTL control, EBV-infected B-lymphocytes may proliferate and develop into B-cell lymphomas (Fig. 55.1). EBV infection also may reactivate into replicative infection at mucosal sites and infectious virus is frequently detected in saliva.

The infected peripheral lymphocytes can be explanted in vitro and established as permanent lymphoblastoid cell lines (LCLs). The majority of the infected cells in the cell lines do not produce virus but instead are latently infected and maintain the EBV genome as a multi-copy episome (Rickinson, 2001). In vitro, an occasional cell in some cell lines may reactivate into viral replication and produce infectious virus. The infectious virus produced by cell lines or virus that is present in throat washings will infect primary B-lymphocytes, establish a latent infection, and induce growth transformation.

The ability of the virus to efficiently transform B-cells in culture to immortalized, transformed cells undoubtedly underlies its connection to human cancers. In B-cell lines grown in vitro, the carefully regulated, coordinate expression of multiple viral gene products induces permanent continuous cell growth.

Characteristics of latent infection

DNA structure

The EBV genome that is encapsidated in the virion is a 185 kb double-stranded, linear DNA molecule that is replicated by a virally encoded DNA polymerase (Kieff and Rickinson, 2001). Within latently infected cells, the viral genome is an extrachromosomal episome that is present in multiple copies (Adams and Lindahl, 1975). The episome has a nucleosomal structure and is replicated by the host DNA polymerase (Shaw, 1985; Shaw *et al.*, 1979). The linear viral genome is largely unique DNA interspersed with short direct tandem repeats. At the ends of the viral DNA there are multiple copies of a 500 bp direct tandem repeated sequence (Given *et al.*, 1979). Due to the variable numbers of this terminal repeat (TR) element the terminal restriction enzyme fragments are heterogeneous in size. Identification of the terminal restriction enzyme fragments of linear virion DNA on Southern blots will reveal ladder arrays of heterogeneous DNA fragments that contain different numbers of TR (Fig. 55.2(a)). After entry into the cell, the linear form circularizes through the TR to form viral episomal DNA. The restriction enzyme fragment representing the fused termini of the episomal form of EBV can be distinguished from the terminal restriction fragments of the linear genomes because the fused fragments are larger and contain the unique DNA sequences adjacent to the terminal repeats from both ends of the genome (Raab-Traub and Flynn, 1986). Therefore the fused termini restriction enzyme fragment will hybridize to DNA probes of unique DNA from either end of the linear genome (Fig. 55.2(a)).

Identification of the EBV termini is particularly useful because it permits discrimination between viral replicative and latent states and also is a predictor of the clonality of the viral genome. In the initial studies, a single band representing the EBV fused termini was detected in NPC and in monoclonal lymphomas (Fig. 55.2(b)). The identification of a single band indicated that all of the EBV episomes were identical to one another with regard to number of TR (Raab-Traub and Flynn, 1986). This indicated that the tumors contained a homogeneous clonal population of EBV genomes and suggested by extension cellular monoclonality. This provided the first evidence that NPC like BL is a monoclonal proliferation and revealed that the malignancy had developed from a single EBV-infected cell. The link between the homogeneity of the EBV genome and cellular clonality has been confirmed in multiple studies of lymphoid tumors where clonality can be determined through analysis of the immunoglobulin joining region (Brown *et al.*, 1988). Most EBV tumors contain a single clonal population of EBV genomes as evidenced by the detection of a single restriction enzyme fragment (Fig. 55.2(b)). Clonal EBV genomes are present in Hodgkin's disease and in many lymphomas that develop in immunosuppressed patients (Weiss *et al.*, 1989a,b). Oral hairy leukoplakia (HLP) is an unusual lesion that may develop on the lateral borders of the tongue in HIV infected or immunosuppressed individuals (Greenspan *et al.*, 1985). It is the only pathologic manifestation of a permissive EBV infection and identification of the EBV termini detects abundant ladder arrays of linear DNA (Fig. 55.2(b)) (Gilligan *et al.*, 1990a).

EBV expression in latent infection

Multiple viral genes are expressed in latently infected lymphocytes maintained in vitro and at least three different patterns of EBV expression have been detected in latently infected cells and tissues (Rowe *et al.*, 1987). In cell lines transformed in vitro and in lymphomas that develop in transplant recipients, six EBNA proteins (EBNA 1, 2, 3A, 3B, 3C, and leader protein, EBNA-LP) are expressed (Rickinson, 2001). These proteins regulate their own expression and also regulate expression of the latent membrane proteins, LMP1 and LMP2 (Wang *et al.*, 1990). In addition, the non-polyadenylated EBER RNAs and rightward transcripts from the BamHI A region are expressed (BARTS) (Brooks *et al.*, 1993). This state of latent infection is termed Type 3 latency. EBV expression is more restricted in other types of latent infections that have been identified in tumors and in peripheral blood lymphocytes. Type 1 latency is the most restricted with expression of EBNA1 and the EBERs and is found in BL. In Type 2 latency found in NPC and HL, EBNA1, LMP1, LMP2, EBERs, and BARTs are expressed. In peripheral blood lymphocytes, EBV may not express any genes or may only express EBNA1 or LMP2. This has been referred to as Type 0 latency (Rickinson, 2001).

The EBNA2 and EBNA3 proteins are the major targets of EBV-specific cytotoxic T lymphocytes (CTL) (Rickinson and Moss, 1997). Expression of these proteins has been detected in tonsillar lymphocytes and during infectious mononucleosis but they have not been detected in latently infected peripheral blood lymphocytes (Babcock and Thorley-Lawson, 2000b). Of the cancers that are associated with EBV, the EBNA2 and 3 proteins are only expressed in lymphomas that develop in immunosuppressed patients such as post-transplant lymphomas (Young *et al.*, 1989). Due to the expression of the EBNA CTL targets, the post-transplant lymphomas can be successfully treated by immune therapy (Rooney *et al.*, 1995) (Fig. 55.1). Reduction

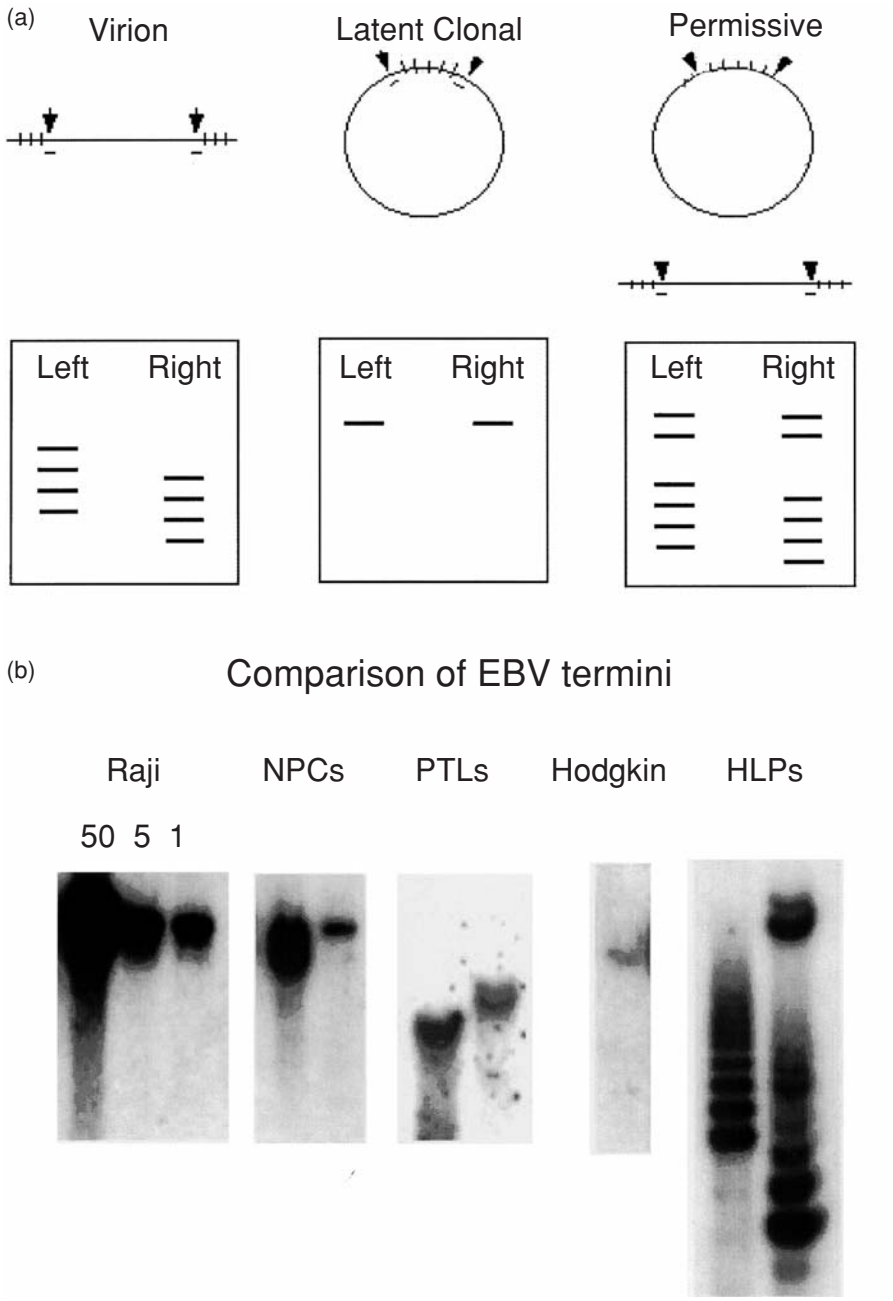

Fig. 55.2. Identification of the EBV termini identifies episomal and linear viral DNA. (a) A is present at the termini of the linear DNA. The number of terminal repeats (TR), homologous direct repeat 500 bp sequence, differs between individual DNA molecules such that the terminal restriction enzyme fragments that contain the TR are heterogeneous in size and form a ladder array on gels representing molecules that have differing numbers of the TR element. Upon entry into the cell, the viral DNA circularizes through the TR to form the intracellular, extrachromosomal episome. This circularization produces a fused restriction enzyme fragment that can be identified with DNA probes representing unique DNA adjacent to the TR from either ends of the linear genome. (b) A single fused restriction enzyme fragment representing the EBV episomal DNA is detected in most EBV malignancies including NPC, PTL, and HD. The Raji cell line and dilutions is used for comparison of copy number. HLP represents a permissive infection.

of immunosuppression or infusion of EBV-specific CTLs that recognize the EBNA proteins can result in regression of the EBV-infected lymphoma. In other EBV associated tumors that develop in non-immunosuppressed individuals and in some patients with AIDS, the EBNA2 and 3 proteins are not expressed (Raab-Traub, 1996). The expression of LMP1 and LMP2 independent of transactivation by the EBNA proteins, the major CTL targets, may be one factor that contributes to the development of these tumors.

The EBNA proteins

The EBNA 1 protein is essential for maintenance and replication of the viral episome by the host DNA polymerase during latent infection (Yates *et al.*, 1985). EBNA1 binds to the origin of replication for the plasmid form of the viral genome and is essential for plasmid replication. EBNA1 also binds to cellular chromosomes and mediates equivalent partitioning of the viral genomes to the daughter cells. Although EBNA1 is expressed in all dividing EBV infected cells, the protein is not recognized by CTLs and the latently infected cells can persist in the presence of a functioning immune system (Levitskaya *et al.*, 1995). The lack of CTL recognition of EBNA1 is due to the presence of a large repeated element of glycine and alanine. This sequence apparently spares EBNA1 from proteosomal degradation and presentation by MHC class I molecules (Levitskaya *et al.*, 1997).

The EBNA 2 protein is essential for growth transformation of lymphocytes and is also a transcriptional transactivator (Cohen *et al.*, 1989). EBNA2 interacts with the RBPJk protein to activate gene expression (Grossman *et al.*, 1994; Henkel *et al.*, 1994). RBPJk is the DNA binding protein and transcription factor that is activated by the Notch signaling pathway. Activated Notch has been shown to partially substitute for EBNA2 in transformation (Hofelmayr *et al.*, 2001). EBNA2 also interacts with multiple cellular transcriptional proteins through which it regulates the viral promoters for the latent membrane proteins, LMP1 and LMP2, for the B-cell activation marker, CD23, and the EBV receptor, CD21 (Johannsen *et al.*, 1995). Two types of EBNA2 have been identified, encoded by quite divergent sequences (Dambaugh *et al.*, 1984). The type of EBNA2 gene, EBNA2A or 2B, distinguishes two types of EBV, EBV1 or 2. EBV1 is more prevalent than EBV2 in Western populations, however, EBV2 infection is prevalent in central Africa, in New Guinea, and among Alaskan Eskimos (Zimber *et al.*, 1986). Coinfection with both EBV types is frequently detected in HIV infected patients and has also been detected in normal individuals (Walling *et al.*, 1992). Recent studies indicate that multiple strains are detected in most individuals (Sitki-Green *et al.*, 2003).

The EBNA3 genes are also encoded by divergent sequences that co-segregate with the EBNA2 type (Sample *et al.*, 1990). Thus the sequence type of the EBNA2 and EBNA3 genes define Type 1 and Type 2 of EBV. All of the EBNA3 proteins interact with the cellular RBPJk protein and may modulate transactivation of the LMP1 promoter and other genes by the EBNA2 protein (Robertson *et al.*, 1996).

Latent membrane protein 1

LMP1 is considered the EBV oncogene as it can transform rodent fibroblasts in vitro and is essential for B-cell transformation (Wang *et al.*, 1985). Transgenic mice that express LMP1 in B cells have increased development of B cell lymphomas (Kulwichit *et al.*, 1998). LMP1 has profound effects on cellular gene expression and induces expression of multiple genes including adhesion molecules, anti-apoptotic functions, growth factors, and growth factor receptors (Wang *et al.*, 1988). The biochemical properties of LMP1 that are in part responsible for these effects on cellular expression are its ability to activate the NFKB transcription factors and to interact with the cellular molecules that mediate signals from the tumor necrosis factor family of receptors (Mosialos *et al.*, 1995). These molecules, entitled TRAFs, form heterotrimeric complexes that transduce signals that depending on the receptor may activate NFKB, induce cellular growth, or induce apoptosis. Thus the activation of the TRAF pathway is likely the key property of LMP1 that mediates its effects on cellular growth regulation.

The LMP1 protein has a short cytoplasmic amino terminus, a six membrane-spanning domain that is responsible for spontaneous aggregation in the plasma membrane, and signaling domains in the carboxyl terminus (Fennewald *et al.*, 1984). The cytoplasmic carboxyl terminus contains two signaling domains, C-terminal activation region 1 (CTAR1) and CTAR2 (Huen *et al.*, 1995). CTAR1 binds TRAFs 1, 2, 3, and 5 while CTAR2 binds the TNF receptor-associated death domain protein (TRADD) and its partner, the TNF receptor-interacting protein (RIP) (Devergne *et al.*, 1996; Izumi *et al.*, 1997). Signaling from these two domains leads to NF-κB activation as well as c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) activation (Eliopoulos and Young, 1998; Eliopoulos *et al.*, 1999). In reporter assays, LMP1 containing CTAR2 but deleted for CTAR1 (LMP1-CTAR2) induces greater NFκB activity, however electrophoretic mobility shift assays (EMSA) indicate that LMP1 deleted for CTAR2 but containing CTAR1 (LMP1-CTAR1) activates at least 3 heterodimeric forms of NFκB including p50/p50, p50/p52, and p52/p65 dimeric forms (Paine *et al.*, 1995;

Miller *et al.*, 1998). These forms are more abundant than the p50/p65 form that is the predominant form of NFκB activated by CTAR2. EBV containing LMP1-CTAR1 is also capable of transforming lymphocytes (Kaye *et al.*, 1999).

Expression of LMP1 affects different cellular genes in lymphocytes and epithelial cells. In both cell types, NFKB transcription factors are activated although different forms of NFKB are activated in the two cell types. Activation of NFKB increases expression of B-cell activation markers in lymphoid cells, and expression of the A20 gene in both lymphoid and epithelial cells. In epithelial cells, LMP1 activates transcription of the epidermal growth factor receptor (EGFR), which is also detected at high levels in NPC (Miller *et al.*, 1995b). Several studies have shown LMP1-CTAR1 has unique properties including the ability to induce expression of the EGFR, TRAF1, and CD54 (Miller *et al.*, 1997; Devergne *et al.*, 1998). The ability of CTAR1 to activate NFκB p50/p50 homodimers may be responsible for its unique ability to induce EGFR expression. Chromatin precipitation assays of the EGFR promoter in NPC have detected 50/p50 homodimers in a complex with bcl3 (Thornburg *et al.*, 2003). It is presently unknown if EBV infection affects bcl3 activity or if the activation of bcl3 is a unique event in NPC.

Latent membrane protein 2 (LMP2)

The LMP2 proteins, LMP2A and LMP2B, are expressed in transformed lymphocytes and in NPC and HL (Young *et al.*, 1991; Busson *et al.*, 1992). The LMP2 gene contains exons located at both ends of the linear EBV genome and can be only transcribed across the fused termini of the episomal form or in some rare integrated events (Laux *et al.*, 1988; Sample *et al.*, 1989). Two distinct forms of mRNA encodes the 54 kd and 40 kd LMP2A and 2B proteins. The 2.3 kb and 2.0 kb RNAs have different promoters and the two RNAs each have a unique first exon and share exons 2 to 9 (Sample *et al.*, 1989). The first exon of LMP2A (exon 1A) encodes the hydrophilic N-terminus whereas the first exon of the B form (exon 1B) is non-coding.

Genetic studies have revealed that LMP2A and B are not required for EBV dependent-transformation of B-cells, however, LMP2A is an important factor in maintaining EBV in a non-replicative state (Longnecker and Miller, 1990). This is accomplished by its ability to alter signaling from the B-cell receptor (BCR), to keep the infected lymphocytes in a non-activated state and maintain a latent infection in B-cells . LMP2 is an integral membrane protein with 12 transmembrane spanning regions. The amino terminal domain that is unique to LMP2A is cytoplasmic and contains multiple potential protein recognition sequences and signaling domains. The motifs include an immunoreceptor tyrosine-based activation motif (ITAM) with two spaced tyrosine residues. This motif is recognized by the Lyn/Syk kinases to transduce BCR signaling (Miller *et al.*, 1995a). LMP2 also contains a PY motif that interacts with the NEDD4 family of ubiquitin ligases (Longnecker *et al.*, 2000; Ikeda *et al.*, 2001). LMP2 apparently sequesters the lyn kinase within lipid rich rafts and promotes its degradation thus blocking BCR signaling and B-cell activation (Dykstra *et al.*, 2001).

Studies in transgenic mice have revealed that LMP2 also provides a cell survival signal (Caldwell *et al.*, 1998). A recent study, using microarray analysis, revealed that LMP2 interferes with expression of specific transcription factors that regulate B-cell development resulting in enhancement of cellular survival (Portis and Longnecker, 2003). In studies in the HaCat epithelial cell line, LMP2 induced growth in soft agar and inhibited differentiation through activation of PI3 kinase and the Akt kinase (Scholle *et al.*, 2000). LMP2 also activates Akt in lymphocytes (Swart *et al.*, 2000). One target of the activated Akt in epithelial cells is GSK3β, an important component in the Wnt/β-catenin signaling pathway. GSK3β exists in a cytoplasmic complex with APC and Axin and it is within this complex that GSK3β phosphorylates β-catenin leading to ubiquitination and degradation of β-catenin by the proteosome complex. GSK3β is inactivated by phosphorylation by Akt (Chen *et al.*, 2000; Cross *et al.*, 1995). Therefore Akt activation may inhibit phosphorylation of β-catenin resulting in its stabilization. In epithelial cells expression of LMP2 greatly increases the levels of β-catenin and also induces its nuclear translocation (Morrison *et al.*, 2003). This property requires activation of Akt and phosphorylation of GSK3. Activation of β-catenin occurs frequently in the development of carcinoma frequently through genetic mutations and it is likely that activation of this pathway is an important factor in EBV effects on epithelial cell growth.

The powerful effects of LMP2 on epithelial cell growth are also suggested by a recent study that revealed that EBV infected epithelial cell clones rapidly emerged from primary infected cultures (Moody *et al.*, 2003). This growth advantage was linked to those clones that had fewer numbers of TR, a property that resulted in increased expression of LMP2.

EBV-encoded RNAs (EBER)

In EBV infected cells, two small non-polyadenylated RNA molecules designated EBER 1 and EBER 2 are transcribed by RNA polymerase III (Lerner *et al.*, 1981). These 170 bp RNAs are encoded by adjacent sequences that have considerable homology. The EBERs exist as ribonucleoprotein complexes and are the most abundantly expressed viral transcript with 10^5 or 10^6 copies per infected cell (Howe and Steitz, 1986). Genetic studies indicate that EBER

expression is not essential for lymphocyte transformation and their function remains obscure (Swaminathan *et al.*, 1991). The Akata BL cell line is unique in that it can be cured of EBV infection. The loss of EBV results in the loss of the ability of Akata cells to form tumors in nude mice (Komano *et al.*, 1998). Expression of EBERS partially revert this loss, potentially through effects on bcl2 (Komano *et al.*, 1999). Similar studies of an EBV infected gastric epithelial cell line have shown that the EBERS induce altered growth properties through induction of expression of IGF1 (Iwakiri *et al.*, 2003).

The extraordinary abundance and stability of the EBER ribonucleoprotein complexes in embedded sections makes in situ detection of EBER expression a useful diagnostic tool to identify EBV-associated diseases (Ambinder and Mann, 1994). However, the EBERs are not expressed at all times in all cells and are not expressed in all examples of EBV infection (Gilligan *et al.*, 1990a; Pathmanathan *et al.*, 1995a). Thus the absence of EBER expression does not necessarily indicate that the lesion does not contain EBV (Niedobitek *et al.*, 1991a,b). The EBERs are not expressed in the differentiated form of NPC or in oral hairy leukoplakia (HLP), a site of EBV replication that contains viral particles, replicative gene products, and linear viral DNA (Gilligan *et al.*, 1990a). The lack of EBER expression in HLP revealed that EBERs were not required for viral replication and indicated that the detection of EBER expression denotes latent EBV infection.

BamHI A transcripts (BARTS)

The BamH A RNAs were initially identified by cDNA cloning of EBV RNAS expressed in NPC (Gilligan *et al.*, 1990b; Hitt *et al.*, 1989). These RNAs are transcribed rightward through the BamHI A fragments (BARTs) and are antisense to multiple replicative functions. At least three mRNAs are abundantly expressed in NPC and consistently detected on Northern blots, while in lymphoid cell lines, these RNAs are only detectable by PCR amplification of cDNAs (RT-PCR) (Gilligan *et al.*, 1990b; Brooks *et al.*, 1993; Sadler and Raab-Traub, 1995b).

In NPC the RNAs are also readily detectable by in situ hybridizations and are transcribed in all cells (Gilligan *et al.*, 1990b). The cDNA clones contain distinct exons and patterns of alternate splicing that produce previously unidentified open reading frames (ORFs) (Sadler and Raab-Traub, 1995b). These cDNAs have been detected by RT-PCR in NPC, BL, parotid carcinoma, peripheral blood lymphocytes, and LCL samples. At the 3′ end of all of the mRNAs is an ORF that would encode a 174 amino acid (aa) protein, *B*amHI *A r*ightward *f*rame *0* or BARF0. This is an unusual ORF with the translational stop codon embedded in the polyadenylation signal (Sadler and Raab-Traub, 1995b). An alternatively spliced cDNA, RK-BARF0, was identified that extended the BARFO ORF from 174 to 279 codons and would encode a protein of approximately 30 kDa. This splice was detected in the least abundant mRNA. The amino terminus of RK-BARF0 protein contains a highly hydrophobic region that resembles an endoplasmic reticulum targeting signal peptide sequence. In yeast two hybrid studies, RK-BARF0 and BARF0 interacted with the extracellular domain of Notch 3 and Notch 4 (Kusano and Raab-Traub, 2001).

Notch defines a family of transmembrane receptor proteins found in a variety of organisms including mammals. Notch is synthesized in the endoplasmic reticulum (ER), and further processed in the trans-Golgi network (TGN) to produce two fragments which are then linked through disulfide bonds (Blaumueller *et al.*, 1997). After ligand binding, Notch is proteolytically cleaved releaving the carboxy terminal domains. This activated intracellular form of Notch is then thought to activate effector molecules, such as the DNA binding protein, RBP-Jk (Fortini, 1994). Activated Notch and RBP-Jk activate expression of several mammalian genes including erbB2a and NF-κB2 and as previously described, the EBNA2 and EBNA3 proteins interact with RBP-Jk to activate expression of LMP1 (Henkel *et al.*, 1994; Grossman *et al.*, 1994). The direct interaction of RK-BARF0 with Notch induces the proteosomal degradation of Notch. Although the RK-BARF0 protein has not yet been detected, EBV infected cells have very low levels of Notch suggesting that RK-BARF0 may be expressed during EBV infection.

The cDNA representing the most abundant RNA contains the RB2 or A73 ORF (Sadler and Raab-Traub, 1995b). The RB2 ORF is formed from 4 exons and has been shown to interact with the receptor for activated kinases (RACK1) (Smith *et al.*, 2000). The RK103 ORF, also called RPMS1, encodes a potential protein that also interacts with RBP-Jk (Sadler and Raab-Traub, 1995b; Smith *et al.*, 2000). The specific splice that is unique to RPMS1 is also readily detected in peripheral blood lymphocytes (Chen *et al.*, 1999b). Although the protein products for these novel ORFs have not been identified, the intriguing properties of the possible proteins suggests that these complex RNAs may encode additional proteins expressed in latent infection in epithelial cells or in B-lymphocytes in vivo.

Malignancies associated with EBV

The malignancies associated with EBV are distinct cancers that develop in the setting of immunosuppression, occur with endemic patterns of incidence, or are a discrete

subset of more common cancers. The most consistent and significant associations include post-transplant lymphoma, African Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma (Raab-Traub, 1996).

Post-transplant lymphoproliferative disease

The ability of EBV to cause cancer is most clearly indicated by the development of B-cell lymphoproliferations in patients who are deficient in T-cell mediated immunity following bone marrow or solid organ transplantation. The majority of cases of post-transplant lymphoma (PTL) are EBV-positive. These proliferations are initially polyclonal but frequently progress to oligoclonal or monoclonal lymphoma and clonal or bi-clonal EBV is readily detected (Fig. 55.2). Depending on the degree or regimen for immunosuppression, PTL develops in 5–15% of cardiac transplants, 10% of heart-lung transplants, in 1 to 3% of renal transplants, and approximately 1 to 2% of bone marrow transplants.

EBV infection in post-transplant lymphoma is an example of Type 3 latency that includes expression of the major CTL targets, the EBNA2 and EBNA3 proteins (Young *et al.*, 1989). In some cases, the disease may regress after reduction in immunosuppressive therapy indicating that the cells are still susceptible to EBV-specific cytotoxic T-cells. Adoptive or prophylactic immunotherapy has also been successful (Rooney *et al.*, 1997). However, there is also variability in EBV gene expression and in the monoclonal, more monomorphic post-transplant lymphomas there may be more restricted Type 1 or 2 latency patterns of viral expression. A recent report described an intriguing case of lymphoma that did not respond to CTL infusion. Subsequent analysis revealed that the EBV strain in the tumor contained a deletion in EBNA3B and that the CTL preparation that had been expanded ex vivo and administered was predominantly directed against EBNA3B (Gottschalk *et al.*, 2001). This provided the first evidence of an EBV escape mutant that evaded immune control due to the deletion of a specific protein. EBNA3B has been shown to not be essential for B-cell immortalization in vitro and its loss would provide an immunoselective advantage in vivo (Tomkinson *et al.*, 1993).

Burkitt's lymphoma

EBV was originally detected in cell lines established from the unusual childhood malignancy, Burkitt's lymphoma (BL) (Epstein *et al.*, 1964). Burkitt's lymphoma was first recognized by a British surgeon working in the colonial office in East Africa (Burkitt, 1962b). He described the clinical and epidemiologic features of this tumor and discovered that the tumor occurred with high incidence in an endemic geographic region that was coincident with the endemic malarial belt of Central and East Africa. Fresh tumor biopsies were sent to Dr. Anthony Epstein in London where he succeeded in establishing cell lines. In some lines, herpesvirus particles were detected by electron microscopy. This virus was subsequently shown to be distinct from other known human herpesviruses and subsequent seroepidemiologic studies revealed that EBV infection was widespread in all populations with the majority of adults having antibodies to the virus (Henle and Henle, 1974).

The early seroepidemiologic studies provided additional evidence that EBV was associated with the cancer. In endemic areas, children become infected with EBV during the first two years of life and endemic BL patients had significantly elevated antibody titers to viral antigens, including the viral capsid antigen (VCA) and early replicative functions (early antigen, EA) (Henle *et al.*, 1971). Prospective epidemiologic studies in Uganda indicated that high EBV VCA antibodies preceded the development of the tumor by months or years.

Characteristics of EBV Infection in BL

Although patients with BL have elevated titers to replicative antigens, EBV gene expression in BL is tightly latent and its expression is very restricted (Rickinson, 2001). Most cells express only EBNA1 and the EBERs. This restricted state of expression is only found in BL and was subsequently termed Type 1 latency. The expression of EBNA1 in the absence of the transcriptional transactivator EBNA2 and EBNA3 proteins reflects the use of an alternate promoter for EBNA1 (Sample *et al.*, 1991). This promoter in the BamHI Q fragment, Qp, is regulated by both IRF and STAT transcription factors (Sample *et al.*, 1992; Chen *et al.*, 1999a). The BARTs have also been detected in BL sample by RT-PCR. Recent studies have indicated that expression in BL may be somewhat heterogeneous with some cells expressing LMP1 and the EBNA proteins that are characteristic of Type 3 latency (Niedobitek *et al.*, 1995). EBV expression in BL also changes in vitro and most BL cell lines reactivate into Type 3 latency with expression of the additional EBNAs and the LMPs. In vitro, this change in expression results in an altered growth pattern with rapid growth of clumped cells and elevated expression of lymphocyte activation molecules, adhesion molecules, and HLA antigens (Rowe *et al.*, 1987).

All of the tumors from the endemic areas contain EBV in all of the malignant cells and each tumor contains a clonal form of the EBV episome indicating that the tumor

developed from a single EBV-infected cell (Raab-Traub and Flynn, 1986) (Fig. 55.2).

Contributing factors

The detection of consistent chromosomal rearrangements in many human cancers suggested that these would cause genetic changes that could alter cellular gene's expression or function. BL was one of the first cancers to be shown to have a characteristic chromosomal translocation and these translocations were eventually shown to involve chromosomal rearrangments of the c-myc oncogene with the loci that encode the immunoglobulin heavy and light chains (Dalla-Favera *et al.*, 1982; Magrath, 1990). This rearrangement induced the aberrant activation of the c-myc oncogene, a critical step in the control of cellular proliferation.

Rare childhood lymphomas in Western countries were subsequently shown to resemble Burkitt's lymphoma histologically. These tumors occur 100-fold less frequently than the endemic form but also are marked by the characteristic translocations between c-myc and the immunoglobulin loci. It is intriguing that the sporadic and endemic forms of BL have different breakpoints with regard to c-myc (Pelicci *et al.*, 1986). The endemic form has breakpoints many kb 5′ to c-myc while the breakpoint in the sporadic form usually occurs in the first exon or intron (Magrath, 1990). These differences in the translocation break point may reflect an effect of EBV in the development of the translocation.

Deregulated c-myc expression is found in all forms of BL suggesting that this is an essential step in the development of this lymphoma. As the translocations involve the immunoglobulin loci, it is possible that they occur during variable gene rearrangement or class switching. The cell surface markers, CD10 and CD77, which are characteristic of BL suggest that BL cells may represent a germinal center B-cell (Rickinson, 2001). Germinal centers are greatly expanded in chronic malarial infection and during HIV infection. The elevated titer to EBV replicative antigens that are characteristic of BL suggest that EBV reactivation and replication has occurred. The activation of viral replication and the expansion of germinal centers are likely contributing factors to the increased risk of development of BL in areas of endemic malaria.

AIDS-associated lymphoma

Lymphomas develop in approximately 3% of AIDS patients and the incidence of BL is greatly increased in HIV-infected patients (Ambinder, 2001). Although in HIV infection, many types of lymphoid malignancies may develop, BL tends to develop early in the course of AIDS progression. The tumors resemble classical BL histologically and also possess the characteristic translocations with the chromosomal breakpoints similar to those in the sporadic BL.

Other B-cell malignancies also occur at high incidence in AIDS and approximately 50% of these are EBV-associated. Central nervous system lymphoma is extremely rare in the general population but occurs in 0.5% of patients with AIDS. All of the CNS lymphomas are EBV positive (MacMahon *et al.*, 1992). Possible genetic or environmental factors that contribute to the development of EBV-positive lymphoma or CNS lymphoma have not yet been identified although the effect of HIV infection on the cytokine network may influence lymphoma development. Both IL6 and IL10 are elevated during HIV infection and promote the growth of EBV-infected B cells (Stewart *et al.*, 1994). In addition to the reduced immune control during HIV infection, abnormal synthesis of these cytokines could activate paracrine or autocrine pathways that influence the outgrowth of EBV-infected cells and promote lymphoma development.

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is an epithelial tumor, that similarly to BL, is characterized by marked geographic and population differences in incidence (Raab-Traub, 2002). It develops with high incidence in southern China and southeast Asia where it may represent 20% of all cancer cases, occurring at a rate of $100/10^5$/year in some regions. The tumor also frequently develops in Eskimo populations and occurs with elevated incidence in Mediterranean Africa. The tumor occurs rarely in Caucasian population, however, unlike BL, all cases of NPC that develop in endemic or nonendemic regions contain EBV (Raab-Traub *et al.*, 1987).

Early seroepidemiologic studies revealed that, similar to patients with BL, patients with NPC had elevated antibody titers to the replicative antigens, VCA and EA. However, NPC patients uniquely had elevated IgA antibodies to these antigens. Detection of IgA antibodies to EBV precedes the development of NPC by several years and the levels also correlate with tumor burden and recurrence (Henle and Henle, 1974). These data suggest that EBV reactivation and replication precede the development of NPC while the induction of IgA antibodies indicates that the replication is occurring at mucosal surfaces.

Subsequent studies revealed that viral DNA and the EBV nuclear antigen, EBNA, were detected in the malignant epithelial cells rather than in the abundant infiltrating lymphoid cells (Wolf *et al.*, 1973). This was the first detection of EBV within epithelial cells.

The tumor presents with varying degrees of differentiation and has been classified by the World Health

Organization into three categories (Shanmugaratnam, 1978). Squamous cell carcinomas, WHO1 tumors, are highly differentiated with characteristic epithelial growth patterns and keratin filaments. Non-keratinizing WHO2 carcinomas retain epithelial cell shape and growth patterns. Undifferentiated carcinomas, WHO3, do not produce keratin and lack a distinctive growth pattern. In addition many tumors have mixed degrees of differentiation or may present as WHO3 at the primary site with increased differentiation in metastatic lymph nodes. The WHO2 and WHO3 tumors have elevated IgG and IgA titers to VCA and EA whereas the WHO1 tumors have EBV serologic profiles similar to control populations. This initially suggested that EBV was only associated with the WHO II and III types. However, subsequent studies have also consistently detected EBV in WHO1 tumors from the Orient (Pathmanathan *et al.*, 1995a). However, in other studies, EBV was not detected in WHO1 NPC from some areas (Niedobitek *et al.*, 1991a,b). The consistent detection of EBV in most NPC from both endemic and non-endemic areas suggests that EBV is an essential cofactor in the development of this tumor.

Characteristics of EBV infection in NPC

EBV infection in NPC is primarily latent with expression of replicative antigens in some cells. Identification of the EBV terminal restriction enzyme fragments in NPC revealed that the EBV genomes were homogeneous and clonal with faint ladder arrays representing linear DNA (Fig. 55.2(b)) (Raab-Traub and Flynn, 1986). This confirmed that EBV infection was predominantly latent and suggested that the tumor was also clonal. The detection of linear DNA also indicated that the tumor was likely the site of the antigenic stimulus for the elevated antibody responses to EBV replicative antigens.

Initial studies of viral expression in NPC revealed striking differences in comparison with EBV expression in transformed B cells. The sequences that were subsequently shown to encode EBNA2 and the EBNA3 proteins were not transcribed and there was abundant transcription from the BamHI A and EcoDhet fragments (Raab-Traub *et al.*, 1983). This pattern of expression was subsequently classified Type 2 latency with expression of EBNA1 from the Qp promoter and transcription of LMP1 and LMP2 (Nonkwelo *et al.*, 1996). Two distinct LMP1 mRNAs were identified in NPC and included the standard 2.8 kb mRNA transcribed from the LMP1 EDL1 promoter and a larger 3.7 kb mRNA that initiated within the TR (Gilligan *et al.*, 1990b; Sadler and Raab-Traub, 1995a). This mRNA has been shown to be regulated by SP1 and STAT3 transcription factors (Sadler and Raab-Traub, 1995a; Chen *et al.*, 2001). LMP1 has been suggested to activate STAT signaling and activated nuclear STAT was detected in NPC. This may indicate that LMP1 in NPC is auto-regulated and that perhaps constitutive STAT activation is a factor that leads to tumor development (Chen *et al.*, 2003).

The abundant transcription from the Bam HI A fragment was further characterized and shown to encode a family of 3′ co-terminal, intricately spliced transcripts (BARTs) that contain multiple potential ORFs (Sadler and Raab-Traub, 1995b). The putative protein products of these ORFs have properties that could in part substitute for the EBNA proteins. RK103/RPMS1 interacts with a co-repressor of RBPJk and negatively affects Notch signaling (Zhang *et al.*, 2001). RKBARF0 also increases LMP1 expression and induces Notch degradation (Kusano and Raab-Traub, 2001). Although Notch inhibits differentiation in some cell types, in epithelial cells Notch induces differentiation. The identification of multiple effects on the Notch pathway by the putative BamHI A proteins and the EBNA proteins suggests that EBV usurps this pathway in both lymphoid and epithelial cells.

EBV infection has been shown to be an early event in the development of NPC and has been identified in rare examples of high grade dysplasia and isolated carcinoma in situ (CIS). In an initial survey, the lesions were extremely rare (11/1798 or 0.6%) with coexistent nasopharyngeal intraepithelial neoplasia with invasive cancer in approximately 3% (58/1798). This extreme rarity of lesions without concomitant carcinoma and the development of invasive carcinoma within 1 year indicated a rapid progression of the initiated cell from dysplasia to carcinoma in situ and invasive cancer (Pathmanathan *et al.*, 1995b). Thus the biologic behavior of NPC is quite distinct from that observed in the malignant progression of mammary carcinoma or cervical cancer where intraductal cancer of the breast or carcinoma in situ of the cervix may persist for years. All cells in examples of CIS expressed EBERs and LMP1 revealing that the lesions were homogeneously infected. Clonal EBV episomes were detected without detectable linear forms of the genome indicating that the neoplasias were a predominantly latent infection. The presence of a single clonal form of EBV implies that the hyperplasia or dysplasia represents a focus of EBV-induced cellular proliferation (Pathmanathan *et al.*, 1995b). One extensive study in high-risk Chinese populations revealed that in early dysplasia EBV was present in a subset of cells while EBV infection in high grade dysplasia was homogeneous and uniform. These studies suggest that an early genetic change precedes EBV infection and induces dyplastic growth and that the EBV infected cells apparently overtake the population and the lesion rapidly progress to CIS (Yeung *et al.*, 1993). A genetic

factor may also influence the ability of EBV to establish a latent infection in epithelial cells rather than replicate as is believed to occur in normal oropharyngeal cells (Fig. 55.1).

During primary infection and during reactivated infection, oropharyngeal epithelial cells with evidence of viral replication have been detected and are thought to be the source for viral shedding (Sixbey *et al.*, 1984). The high IgA titers to EBV replicative antigens that precede the development of NPC patients to VCA likely reflect increased viral replication and antigenic stimulation. The elevated IgA titers may actually contribute to the development of NPC by enhancing epithelial infection as it has been shown that secretory IgA facilitates entry into epithelial cells (Gan *et al.*, 1997). In the development of NPC, increased viral replication, perhaps at lymphoid/mucosal epithelial interfaces, increases the likelihood of establishing a latent, transforming infection of an epithelial cell that may have already had an initiating genetic change. This establishment of latent infection and expression of critical viral transforming functions in an epithelial cell in combination with genetic changes that may facilitate latent infection or are synergistic with EBV transforming proteins are likely to be critical events that lead to the development of NPC. This process could be influenced by the genetic or environmental factors (Fig. 55.1). However, the viral transforming functions are likely essential to the malignant outgrowth.

Contributing factors

The incidence of NPC is low in Western populations where it is only 0.25% of all cancers occurring with a rate of $0.1/10^5$ per yr. However in contrast to the relationship of EBV to Burkitt's lymphoma, EBV is consistently detected in NPC regardless of geographic distribution or the racial background (Yu and Yuan, 2002). The consistent association with a ubiquitous herpesvirus and the remarkable patterns of incidence suggests that other genetic or environmental factors contribute to tumor development.

Comparisons of EBV infection among Indians and Chinese living in Singapore revealed that both populations were infected with EBV early in life, usually between the ages of 6 to 9 (Shanmugaratnam, 1970). Although the two ethnic groups were living in the same general area of Singapore, the NPC incidence was only high in the Chinese population. Elevated incidence is retained by second generation Chinese in other nonendemic regions but gradually decreases with subsequent generations (Buell, 1974). This continued elevated incidence suggested that environmental contaminants were not likely to be a cofactor but rather that genetic or cultural and dietary differences contributed to the development of this disease. One dietary component that has been suggested is exposure to salted fish at an early age (Ho, 1976; Yu *et al.*, 1986). Tumor promoting compounds have also been identified in food products in other populations with elevated incidence (Poirier *et al.*, 1989).

Extensive surveys for activated oncogenes or tumor suppressor loss did not reveal characteristic translocations, mutations in p53 or Rb alterations, or activating ras mutations (Effert *et al.*, 1992; Sun *et al.*, 1995, 1993). This suggests that viral genes affect these pathways, directly or indirectly, such that there is no selection for mutation in these genes (Fries *et al.*, 1996). However, additional genetic changes could develop during tumor growth and contribute to tumor progression and metastasis.

To identify potential tumor suppressor genes, screening of NPC samples has detected specific loss of of DNA sequences indicated by loss of heterozygosity in PCR screenings. This approach has shown that the p16 cyclin dependent kinase inhibitor at 9p21 and the RASSF1a gene at 3p14 are frequently lost in NPC samples (Lo, 2002).

Studies in vitro have shown that reintroduction of the RASSF1a gene into an NPC cell line inhibited growth (Lo, and Huang 2002). This finding suggests that inactivation of this ras isoform is an important contributor to NPC development. Other studies have shown that p16 is not expressed in NPC due to specific methylation of the promoter. In vitro studies have shown that LMP1 expression induces methylation of the p16 promoter and that LMP1 also induces cytoplasmic translocation of the ets transcription factor that regulates p16 promoter activity (Ohtani *et al.*, 2003). The loss of p16 may be highly synergistic with LMP1 expression in the induction of NPC.

Hodgkin's lymphoma

Hodgkin's lymphoma (HL) is a common malignant lymphoma characterized by the loss of lymph node architecture with the majority of infiltrating cells of a nonmalignant phenotype. The malignant cells are the unusual Hodgkin and Reed–Sternberg (RS) cells that constitute about 2% of the tumor mass (Ambinder *et al.*, 1999). Dependent on the ratio of RS cells and the type of infiltrate, HL is histologically distinguished as lymphocyte-predominant, nodular sclerosing, mixed cellularity, or lymphocyte depleted. EBV is always found in the lymphocyte depleted form, approximately 70% of mixed cellularity, 10%–40% of nodular sclerosing and never in lymphocyte enriched (Chapman and Rickinson, 1998).

The disease occurs world-wide but has higher incidence in higher socio-economic groups in Western populations where it occurs with two peaks at ages 25 to 30 or again later than age 45. It was observed years ago that the profile of the

early age onset group was similar to patients who developed infectious mononucleosis, the pathologic manifestation occurring in post-adolescent primary EBV infection. A history of IM was noted to be associated with a 2–4 fold increased risk for HL. Retrospective analyses of the serum repository at Yale revealed that elevated EBV titers preceded the development of HL by 2–3 years (Mueller *et al.*, 1989).

The key finding revealing an etiologic association of EBV with HL was the detection of the viral genome and virally encoded proteins in the RS cells (Weiss *et al.*, 1989a,b). Again, the analysis of the EBV genome revealed that HL had clonal EBV episomes, indicating that HL develops from an EBV-infected cell (Fig. 55.2(b)). Monoclonality has also been revealed by immunoglobulin gene rearrangements and in some cases, T-cell receptor rearrangements. The tumors predominantly have expression of B cell markers although T-cell receptor rearrangement has been detected in some cases (Marafioti *et al.*, 2000).

Transcriptional studies have shown that HL has expression of EBNA1, LMP1, LMP2 and the EBERS, indicative of Type 2 latency (Deacon *et al.*, 1993). Several of the genes known to be activated by LMP1 are expressed in HL and activated NFκB has been found in the RS cells (Hinz *et al.*, 2002). The same forms of activated NFκB have been detected in EBV negative RS cells (Knecht *et al.*, 1999). In the EBV negative examples of HL, mutations have been described within the IκB repressor of NFκB (Emmerich *et al.*, 2003). This finding suggests that activation of NFκB is likely an important factor in the development of HD and that it occurs either via LMP1 expression or by other mechanisms.

Although HL frequently responds well to treatment, new methods of targeting EBV infection have been attempted (Bollard *et al.*, 2004). CTL therapy has had some success and new trials are testing inhibitors of NFκB.

T-cell lymphoma

Although long believed to be B-cell trophic, EBV has been detected in an occasional T-cell lymphoma. A particular type of T-cell lymphoma that usually presents in the nasal cavity, also referred to as midline granuloma, is a common tumor in southeast Asians. The first link to EBV was presented in five Japanese cases, all of which were EBV-positive (Harabuchi *et al.*, 1996). The tumors had various T or NK cell markers suggesting EBV infection of a peculiar undifferentiated cell type.

Peripheral T-cell lymphomas that are EBV-positive have also been described in Taiwanese and Japanese populations. In some cases, EBV was only detected in some cells suggesting that the virus infected the tumor secondarily (Kanegane *et al.*, 1999). However, the proportion of EBV infected cells seems to increase over time with emergence of a clonal EBV-infected population indicating that the virally infected cells have some growth advantage and that the fastest growing clone will eventually predominate.

Rare carcinomas

Other undifferentiated carcinomas that develop in other tissues are also associated with EBV. Clonal EBV episomes have been detected in all samples examined of undifferentiated carcinoma of the parotid gland (Raab-Traub *et al.*, 1991). These studies revealed that carcinoma of the parotid gland is also a clonal proliferation of non-permissively infected epithelial cells. Viral shedding is believed to occur in epithelial cells lining the parotid gland without apparent pathology. The establishment of a latent transforming infection in these cells is apparently a rare occurrence that contributes to the development of this tumor. Undifferentiated carcinoma of the parotid gland is an extremely rare cancer that has been most often detected in Alaskan Inuit populations who also have a high incidence of NPC (Lanier *et al.*, 1991).

EBV infection has also been detected in undifferentiated gastric carcinoma, a rare tumor in both Oriental and Caucasian populations (Shibata *et al.*, 1991). These findings indicate that EBV may gain access to epithelial cells outside the naso/oropharynx and that in some instances when this occurs, it leads to the development of carcinoma.

Contributing factors

Genetic factors

Tumor suppressor inactivation

The development of cancer involves multiple oncogenic events and several possibly contributing genetic components have been identified in EBV associated cancers. Loss of heterozygosity (LOH) is a highly informative approach for detecting allelic deletion of potential tumor suppressor functions. This approach has been used most successfully in solid tumors such as NPC. These studies have determined that the highest frequencies of allelic loss occurred on chromosome 3p and 9p (Huang *et al.*, 1991, 1994; Lo and Huang, 2002). Intensive efforts have localized the region on 3p to 3p21.3. Functional evidence of tumor suppressor function within this region have shown that chromosomal transfer into an NPC cell line abrogated growth. The target gene has been demonstrated to be an allelic form of

RASSF1A, a newly discovered gene that contains a ras association domain (Cheng *et al.*, 1998). Thus RASSF1A may modulate ras function.

The site of LOH at 9p21 spans the cyclin-dependent kinase inhibitors (cdki) p15INK4B and p16INK4A and the negative regulator of p53, p14ARF . Loss of p16 has been shown in NPC samples and transfer of p16 induced growth arrest in the NPC cell line HK1 (Lo *et al.*, 1995). Homozygous deletion and promoter methylation of p14 ARF has also been demonstrated in many NPC tumors. The loss of this locus has been shown to occur early in dysplastic nasopharyngeal cells (Lo and Huang, 2002). The loss of this locus in combination with EBV infection likely contributes to the rapid development of invasive NPC.

Similarly, the promoters for p15 and p16 have been shown to be methylated in Hodgkin's lymphoma suggesting that loss of cdki function is also a contributing factor in EBV-associated lymphoid diseases (Garcia *et al.*, 2003).

EBV strain variation

The elevated incidence of EBV-associated malignancies in specific populations suggests that one possibility for these differences in disease incidence may be difference in prevalence of EBV variants. These strains could possess distinct biologic properties or be less immunogenic. The prevalence of specific strains within a population could contribute to these differences in disease incidence. EBV types 1 and 2 are distinguished by sequence changes in the EBV nuclear antigens 2 and 3 (EBNA 2 and 3) and several variants of EBV have been identified by polymorphisms in the viral genome (Abdel-Hamid *et al.*, 1992; Khanim *et al.*, 1996; Lung *et al.*, 1990). Strain specific changes have also been identified in EBNA1, BZLF1, and LMP1 (Miller *et al.*, 1994; Bhatia *et al.*, 1996; Packham *et al.*, 1993; Edwards *et al.*, 1999). The incidence of BL is high in Papua New Guinea with a frequent occurrence of HLA A11. Multiple A11-restricted epitopes have been identified in EBNA3 and analysis of the EBNA3 sequence in the virus prevalent in this area revealed that it contained changes within key positions in the A11 restricted epitopes. These changes and decreased immune recognition may contribute to the prevalence of a given strain within a population and perhaps to a higher viral burden in A11 individuals which might be possible etiologic factor in the development of BL in this region.

Many of these studies have focused on detecting strains possibly unique to NPC. Analyses of strain variation based on restriction enzyme polymorphisms identified a predominant strain in NPC from Southern China (Lung *et al.*, 1990). Various polymorphisms within restriction sites were identified including a loss of an Xho1 restriction enzyme site within the LMP1 gene (Hu *et al.*, 1991). This polymorphism was also present in all NPC from Alaska and in some of the NPC samples from Caucasian Americans but not in samples of NPC from Mediterranean Europe and Africa (Abdel-Hamid *et al.*, 1992).

Further studies of sequence variation within LMP1 revealed that consistent sequence variation distinguished EBV strains and seven phylogenetically distinct strains of LMP1 have been described that have signature changes in the coding sequence (Edwards *et al.*, 1999). Individual isolates differ by these specific base pair (bp) changes, the presence or absence of a 30 bp deletion, the number of 33bp repeats, and a 15 bp insertion within one of the repeats. Strains with or without the deletion have been detected in approximately equal proportions in the various EBV-associated diseases suggesting that all strains can be pathogenic (Miller *et al.*, 1994; Khanim *et al.*, 1996). However, several studies have suggested that LMP1 with the deletion has enhanced transforming potential in vitro and may be present in more aggressive disease forms (Knecht *et al.*, 1992). One study indicated that the enhanced transforming ability of the Chinese strain in BalbC 3T3 cells was transferred with the carboxy terminus that included the 10 aa acid deletion. Deletion of the 10 aa in the B95 LMP1 resulted in the ability to induce transformation and tumorgenicity while insertion of the 10 aa into the Chinese strain eliminated transformation and tumorigenicity (Li *et al.*, 1996).

Heteroduplex tracking assays (HTA) have been useful for distinguishing viral variants and following HIV strain evolution (Nelson *et al.*, 2000). To identify viral variants within a tissue sample, a specific DNA sequence is amplified from the sample, denatured, and reannealed to a single stranded probe of that region (Fig. 55.3(a)). Homoduplexes migrate faster during agarose electrophoresis while the migration of mismatched heteroduplexes is impaired (Fig. 55.3(b)). In the HTA developed for LMP1, the amplified region spans the 30bp deletion (Sitki-Green *et al.*, 2002). Strains are distinguished using a probe that contains the 30bp from the China2 strain (Ch2) and a probe of the same region that spans the deletion (Med-). Probes from two of the strains have been utilized to clearly identify each variant of the LMP1 types, China 1 (Ch1), Mediterranean with the deletion (Med+), Mediterranean without the deletion (Med-), North Carolina (NC) Alaskan (Al), and the prototype, B95. Each form of LMP1 consistently migrates to the same position when hybridized with each probe (Fig. 55.3(b) & 55.3(c)).

Using this approach, HTA revealed that infection with multiple strains of EBV is a frequent occurrence in patients with HIV and also in healthy, asymptomatic carriers

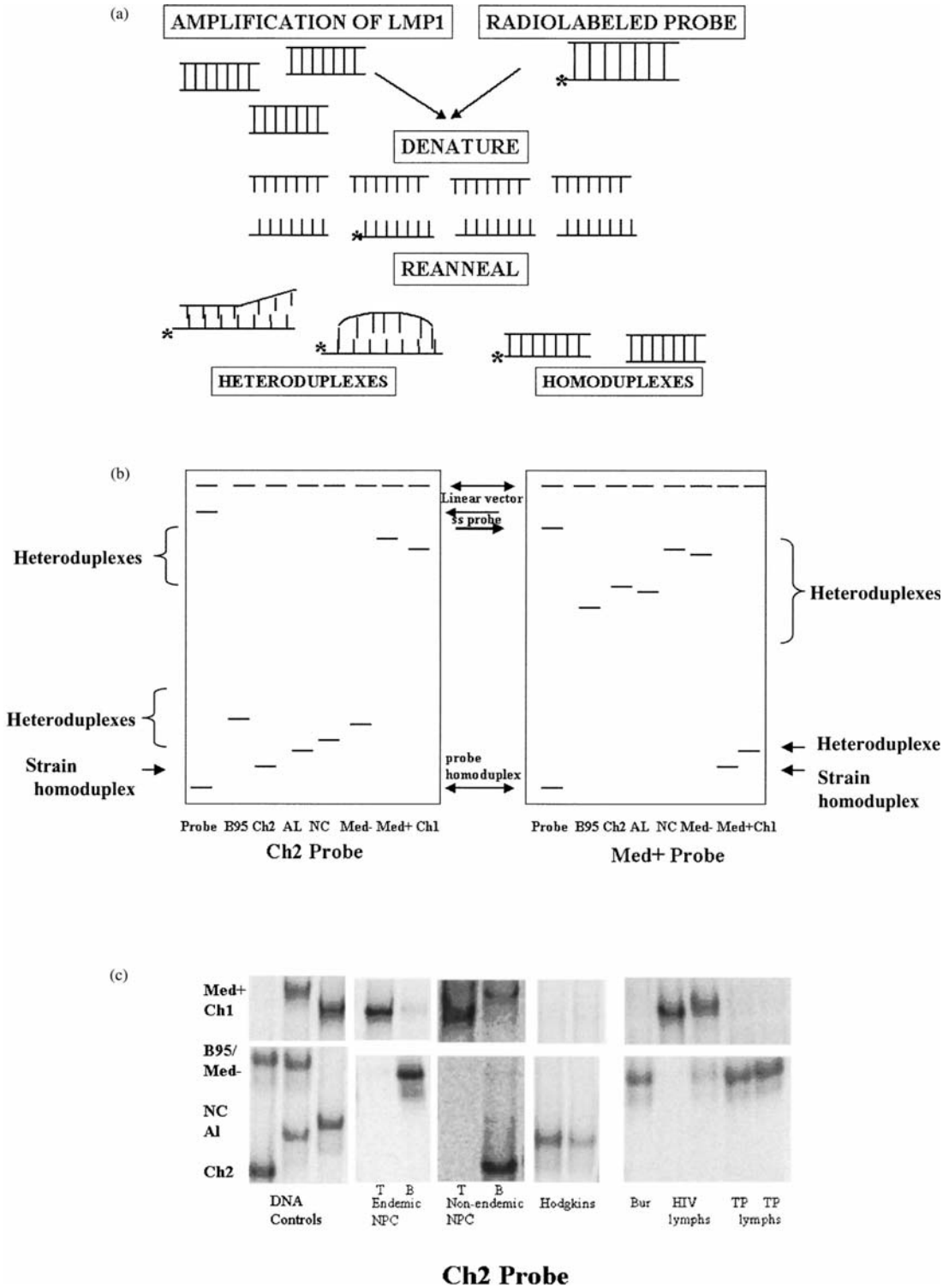

Fig. 55.3. Heteroduplex tracking assay to identify EBV LMP1 variants. (a) HTA analysis is based on the principal that 100% homologous DNA molecules, homoduplexes, will migrate according to size on a polyacrylamide gel, while, heteroduplexes will migrate more slowly because of malformations in the DNA helix corresponding to regions of mismatch. The greater the mismatch of two sequences, the slower the migration of the heteroduplex through a gel matrix. A specific region of LMP1 harboring multiple sequence changes is amplified from each sample, denatured, and hybridized to a single-stranded labeled probe. (b) Diagram of HTA using two probes to form heteroduplexes with each PCR amplified strain that are subjected to electrophoresis through a non-denaturing gel. Distinct migration patterns of heteroduplexes are produced for each strain. (c) HTA analysis reveals single variants in NPC, HD, Burkitt lymphoma (Bur), and HIV and post-transplant (TP) lymphoma samples.

(Sitki-Green *et al.*, 2003). The relative abundance of the strains changes over time with different strains prevalent in the oral cavity than in the peripheral blood at a particular time. HTA analysis of tumor samples has revealed that EBV-associated tumors contain a single strain of EBV while blood and saliva samples usually contain multiple strains of EBV (Fig. 55.3(c)). This provides another perspective of EBV infection in tumors and supports the concept of a homogeneous, clonal infection.

Comparison of the strain prevalence in NPC tumor samples with matching blood samples revealed that the LMP1 variant China1 was almost always in the tumor while other EBV LMP1 variants were present in the blood (Fig. 55.3(c)) (Edwards *et al.*, 2004). In many cases the strain within tumor tissue had changes in the known and computer-predicted HLA restricted epitopes of the HLA type of the patient. Patients with NPC are frequently HLA A2 and A24 and the China 1 strain have been shown to be changed at the strongest A2 epitope. These amino acid substitutions at key positions in these LMP1 epitopes may result in a reduced CTL response that enables immune escape of latently infected cells that express the China 1 LMP1.

The elevated incidence of NPC and BL in restricted populations such as Alaskan Eskimos and Cantonese from Southeastern China may reflect a predominance of a specific MHC type. Higher incidence of a specific MHC haplotype in these populations could have selected for viruses with mutations in putative CTL recognition sequences in LMP-1. The prevalence of such a type in these populations may allow expression of LMP-1 in infected cells to go undetected. Thus a combination of a specific EBV strain in a patient with a particular HLA type could be a contributing factor to tumor development due to decreased immune recognition of a specific LMP1 or perhaps other proteins expressed in the tumor such as LMP2 or the putative BamHI A proteins.

Summary and future considerations

EBV-targeted therapy

The strong association of EBV with major human malignancies provides an opportunity to specifically target EBV or pathways activated by EBV to treat these cancers. A potential vaccine could either protect against infection or possibly eliminate specific pathologic consequences. To prevent viral infection, it would be necessary to completely neutralize EBV at mucosal surfaces through efficient induction of IgA antibodies. This could theoretically be accomplished with a transformation defective EBV that would replicate at mucosal surfaces. Several studies indicate that there are naturally occurring EBV strains that lack the EBNA2 gene and are transformation negative (Sixbey *et al.*, 1991). A genetically engineered strain of EBV that lacked essential transforming proteins yet replicated efficiently at mucosal surfaces might be useful as an attenuated vaccine.

An alternative approach would be a subunit vaccine. The viral glycoprotein gp350, is the most abundant viral glycoprotein and is essential for viral binding and infection. The protective ability of gp350 antibodies has been tested in cotton top marmosets challenged with a lymphoma inducing growth (Finerty *et al.*, 1992). Some protection was provided that seemed to be cell mediated rather than antibody mediated (Wilson *et al.*, 1996). A gp350/vaccinia recombinant was also tested in China in seronegative children. After 1 year, all ten of the control group had seroconverted and had antibodies to VCA, indicating wild-type EBV infection in comparison with the test group where only three had seroconverted (Gu *et al.*, 1995).

A novel approach has developed synthetic peptides representing the predominant CTL epitopes presented by prevalent class I molecules. Expression of a cocktail of EBV epitopes in vaccinia virus indicated that all of the epitopes were processed correctly for the individual HLA classes and that the virus could induce the correct EBV epitope-specific CTLs in vitro (Moss *et al.*, 1996). Many EBV CTL epitopes have been identified and it is possible that an appropriate cocktail could be selected to protect the majority of individuals. It may also be possible to enhance a specific CTL response. In NPC and Hodgkins's disease, the viral proteins which induce the immunodominant CTL response are not expressed. It is possible that the viral expression could be manipulated perhaps through the use of demethylating agents such as azacytidine to induce expression of the viral proteins that would make the cells susceptible to CTL killing (Robertson, 2000). Alternatively, the CTL response to weaker immunogens such as LMP1 or LMP2 could theoretically be enhanced which may enable immune recognition and control of the tumor. Immunotherapy and other approaches to alter EBV expression or induce viral replication are reviewed in other chapters.

The EBV associated cancers are classic examples of the multistep nature of cancer development. The malignancies develop as a combination of a common virus with potent transforming ability, possible immune impairment, increased genetic susceptibility, in part due to HLA type, and genetic changes possibly due to environmental exposure. Potential steps in the pathway to cancer would be some event or exposure that increases the amount of virally infected cells. This might be an increase in viral replication in combination with an expanded population of a specific cell type. Thus in the development of BL or HIV lymphomas,

an expansion of germinal center B-cells due to malaria or HIV and their infection by EBV is likely a contributing early event. Important interactions between the intracellular environment and the virus next occur. These include factors that contribute to establishment of a latent, transforming infection rather than a permissive viral replicative infection and the ability to express LMP1 and LMP2 without expression of the CTL targets, EBNA2 and EBNA3. Important contributing factors include activation of STATs, expression of IL6, and lack of p16 expression. The ability of the growing LMP1-expressing cells to evade immune recognition is critical at this point and specific strains of EBV in certain HLA backgrounds would be able to persist. The EBV transforming proteins would take over multiple cellular signal transduction pathways through direct effects and indirect effects on expression of cellular genes. Multiple other genetic changes may occur during this process and have important effects on cell growth. However, the potent effects of EBV on cell gene expression suggest that the latently infected cells would be rapidly growing and quickly invasive. Importantly, this scenario suggests that EBV and expression of viral proteins are essential to the cancer process, to both initiation and progression. The dependence of tumor growth on viral proteins and activation of specific signaling pathways enables targeting of EBV-associated tumors through immunotherapy and specific inhibitors of activated pathways.

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56

KSHV-induced oncogenesis

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Human infection by KSHV is associated with the development of at least three proliferative disorders: Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and a subset of multicentric Castleman's disease (MCD). In keeping with the classification of KSHV as a lymphotropic ($\gamma 2$) herpesvirus, two of these (PEL and MCD) are primary disorders of the B cell lineage. The third, KS, is a more complex lesion driven by proliferation of cells of endothelial lineage. KSHV is the second human γ-herpesvirus to be linked to neoplasia (EBV being the first). As such, many notions about how KSHV engenders these lesions have been heavily influenced by paradigms derived from the study of EBV-induced malignancies. In EBV, the viral latency program is powerfully immortalizing in vitro, and is thought to be the principal genetic program driving virus-related tumorigenesis. Lytic infection, while presumed important for dissemination of infection to target cells early in infection (and following lytic reactivation at later times), is not thought to play a direct role in the histogenesis of the tumors. As we shall see, although many parallels indeed exist with EBV, the distinctive features of the KSHV-associated diseases makes routine extrapolation from other viral models an enterprise to be undertaken with caution. In this chapter, we will review the biology of the KSHV-associated malignancies and consider the cellular and molecular mechanisms by which KSHV infection contributes to their pathogenesis.

Primary effusion lymphoma (PEL)

PEL is a classical neoplasm involving cells of the B cell lineage (see review by Cesarman, this volume). The malignant cells in PEL are clonal (as judged by VDJ rearrangement; Knowles *et al.*, 1989; Green *et al.*, 1995), fully immortalized, grow readily in culture (Cesarman *et al.*, 1995; Boshoff *et al.*, 1997) and are tumorigenic in nude mice (Picchio *et al.*, 1997). They express few classical B-cell surface markers, but regularly express CD138/syndecan-1 and display somatic hypermutation in Ig and bcl-6 loci, indicating that they are derived from post-germinal center B-cells (Carbone *et al.*, 2001). Consistent with this, transcript profiling suggests that the cells are of plasmablastic lineage (Klein *et al.*, 2003; Jenner *et al.*, 2003). The fact that PEL cells often secrete IL6 and IL10, a feature that is shared with other plasmacyctic neoplasms (Lauta, 2003), also accords with this interpretation.

Virtually all cases of PEL harbor KSHV DNA in every cell; many also display EBV infection as well, though EBV is never found in PEL as the sole pathogen. Where the EBV genome is present, its expression is largely restricted to EBNA 1, EBER RNA and low levels of LMP2a (typical of the so-called Latency I pattern) (Horenstein *et al.*, 1997; Szekely *et al.*, 1998; Schulz, 2001). KSHV infection of PEL cells is predominantly latent, which has made PEL cell lines the most widely studied model for KSHV latency. Most PEL lines also display a small subpopulation of cells staining for markers of lytic reactivation (Staskus *et al.*, 1997; Sturzl *et al.*, 1997). Latently infected PEL cells express at least 5 loci (see below), none of which have been conserved in EBV. Most of these loci were discovered by screening for transcripts or antigens constitutively expressed in PEL cell lines. Their products have been studied intensively for their biochemical and phenotypic properties, usually by overexpression in heterologous cell types. As we shall see, in the aggregate they harbor numerous activities that can deregulate cell growth and augment cell survival, consistent with the notion that KSHV latency drives the generation of PEL. The rarity with which PEL follows KSHV infection, however, also suggests that while viral latency is surely necessary for PEL development, it may not be sufficient for it. Host factors – most likely additional host cell

mutations – must also be involved, but the number and identities of these have not been determined. It is known, however, that PEL cells do not harbor rearrangements of MYC or BCL-2 genes. One study reports that cultured PEL cell lines have frequent reduplications of chromosome 7 and 12, as well as abnormalities at 1q21–25 (Gaidano *et al.*, 1999), but the key genes affected by these processes remain to be identified. In addition, two autocrine or paracrine loops involving host genes have been posited to occur in PEL. One report indicates that both HGF (hepatocyte growth factor) and its receptor (c-Met) are expressed in primary PEL tumors and in PEL cell lines (Capello *et al.*, 2000). More widespread has been the recognition that PEL cells usually express the IL6Rα and gp130 chains that mediate responsiveness to IL6, and often secrete human IL6 itself, raising the possibility of an autocrine loop. However, recent studies suggest that it is a viral homologue of IL6 (v-IL6; see below) that actually functions in this role, together with IL10 (Jones *et al.*, 1999).

The KSHV latency program in PEL

Since both KSHV and EBV are lymphotropic herpesviruses linked to B-cell lymphoma, it is natural to imagine that similar mechanisms must underly their oncogenic programs. However, a closer look reveals many phenotypic differences that challenge this assumption. First, while in vitro EBV infection of primary B-cells is strongly immortalizing, there is little evidence that KSHV infection can immortalize lymphocytes on its own. This issue has been most rigorously addressed by Kliche *et al.* (1998), who showed that under conditions in which EBV efficiently promoted outgrowth of lymphoblastoid cell lines, KSHV failed to do so when the PBMCs were from EBV-negative hosts. Only with B cells from EBV-infected hosts could KSHV infection be linked to B cell outgrowth, and in all cases the resulting LCLs harbored both viral genomes. This would suggest that the KSHV latency program is not strongly immortalizing on its own, and, at least in vitro, either requires one or more cofactors from EBV or acts to stimulate EBV's immortalizing activity. These findings accord well with the finding that most PEL tumors harbor both EBV and KSHV. Nonetheless, it is clear that the *sine qua non* of PEL is KSHV infection, since PEL tumors are not observed in its absence. And since occasional PEL tumors harbor KSHV alone, whatever cofactor(s) are derived from EBV must also be obtainable in some alternative way.

These findings illustrate that KSHV latency must make some genetic contribution to PEL, but do not define the biochemical nature of that contribution or identify the responsible viral gene(s). Two major experimental obstacles have impeded progress on this question. First, KSHV infection of primary B-cells proceeds very inefficiently in vitro, making it difficult to assay biochemical or phenotypic events directly triggered by latency in this lineage. Second, systems for mutating viral genes in the context of the intact KSHV genome are exceedingly inefficient – to date only a few viral genes have been inactivated in this fashion (Delecluse *et al.*, 2001; Xu *et al.*, 2005), though two laboratories have been able to construct genetically marked WT KSHV isolates (Viera *et al.*, 2001; Zhou *et al.*, 2002).

In the absence of such systems, inferences about KSHV's genetic and biochemical contributions to PEL must be derived from studies of the effects of expression of individual latency genes in heterologous cells, usually fibroblasts or other non-lymphoid cells. A previous chapter in this volume described the identification of the viral genes transcribed in latent PEL cell lines; these transcription units and their map positions are pictorially summarized in Fig. 56.1. Here we consider what is known about each of these proteins and how this knowledge might relate to PEL development.

Latency-associated nuclear antigen (LANA)

This large, multifunctional protein is expressed in all latently infected cells in vivo, irrespective of their lineage – it is the universal marker of KSHV latency and is present in KS spindle cells as well as the B-cells of MCD and PEL (Dupin *et al.*, 1999). Studies in Tg mice affirm that its promoter is expressed efficiently in both B-cells and epithelia (Jeong *et al.*, 2002). (Paradoxically, this study failed to demonstrate endothelial expression of LANA, despite its ubiquitous expression in human KS spindle cells. This suggests that either the promoter fragment employed lacked recognition sites for endothelial-specific factors, or there are species differences in LANA gene regulation). The protein has three major domains (Fig. 56.2): a central region composed of variable numbers of highly acidic amino acid repeats, a C-teminal, more basic region involved in DNA binding and oligomerization, and an N-terminal region to which many functions have been ascribed, including chromatin attachment and corepressor recruitment (Krithivas *et al.*, 2000; Piolot *et al.*, 2001; see below). The best-characterized function of LANA is that involved in the establishment and maintenance of the latent viral episome in the nucleus (Ballestas *et al.*, 1999; Cotter and Robertson, 1999). This activity is based upon its ability to (i) bind to sequences in the terminal repeats (TRs) of the genome (Ballestas and Kaye, 2001; Garber *et al.*, 2002); (ii) mediate transient semiconservative DNA replication from TR-containing plasmids (Hu *et al.*, 2002; Lim *et al.*, 2002; Grundhoff and

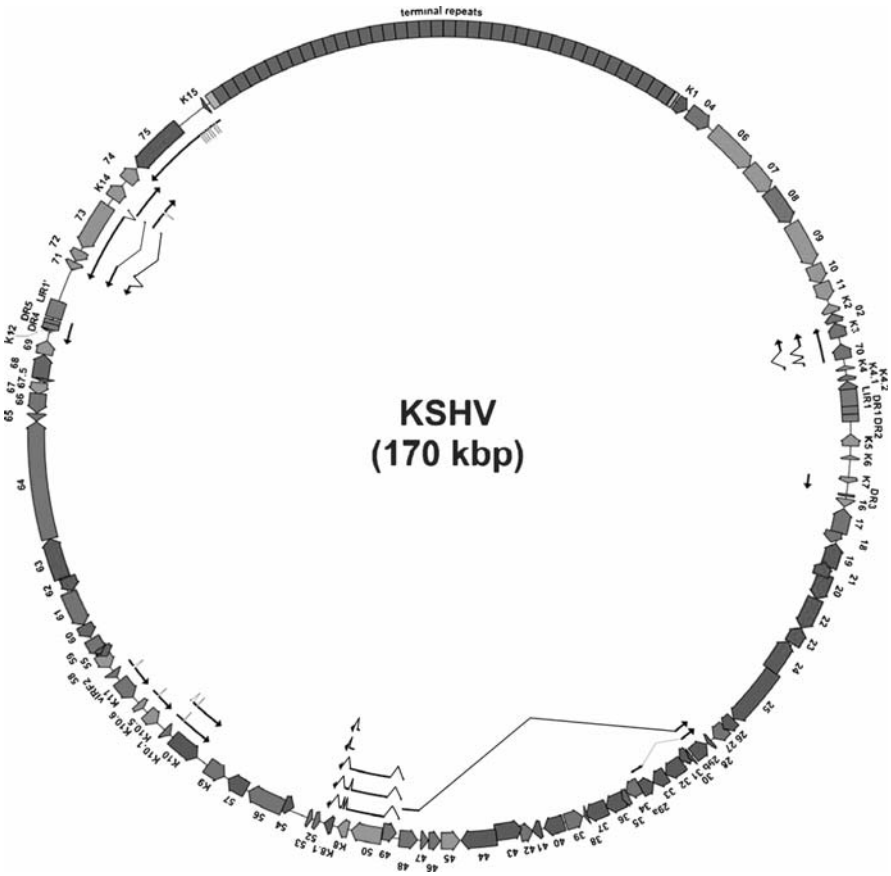

Fig. 56.1. Latency Genes of KSHV. Transcripts of latent genes are depicted as arrows, superimposed on the physical map of the circular, latent viral genome. (See color plate section.)

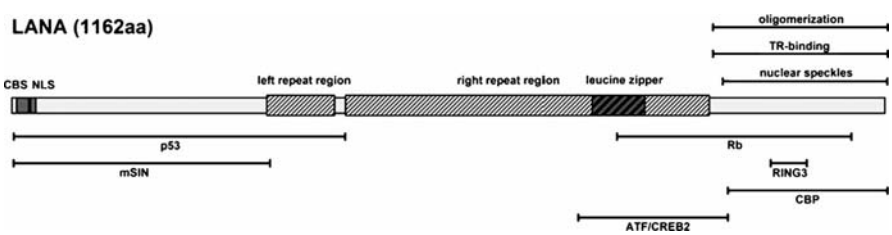

Fig. 56.2. Domain organization of LANA. Regions implicated in binding some of the putative LANA-associated host factors are denoted with lines above or below the name of the corresponding factor. CBS, chromatin binding sequence; NLS, nuclear localization sequence.

Ganem, 2003); and (iii) bind mitotic chromosomes to promote segregation of viral genomes to the progeny of dividing cells (Ballestas *et al.*, 1999; Piolot *et al.*, 2001). However, the story of viral episome maintenance is more complex than this. In many biologically important contexts, while LANA is necessary for plasmid maintenance, it is not always sufficient for it – additional cis-acting epigenetic changes in the viral chromatin appear to be required (Grundhoff and Ganem, 2004). This will be considered more fully in a later section.

Apart from its role in plasmid maintenance, the protein has been suggested to function in several other pathways. Friborg *et al.* (1999) showed that LANA can bind p53, and that cells transfected with LANA displayed reduced activation of p53-dependent reporter genes and increased resistance to p53-dependent programmed cell death

triggered by several stimuli. Such an activity could serve to extend the lifespan of a B-cell, though it seems unlikely (by itself) to be able to account for the B-cell proliferation that characterizes PEL. Although direct tests of this have not been carried out in primary B cells, LANA expression can extend the survival of cultured primary endothelial cells, but it does not fully immortalize them (Watanabe *et al.*, 2003). Similarly, we have found that LANA overexpression in Tg mice in several non-lymphoid lineages does not induce either hyperplasia or neoplasia (Grundhoff and Ganem, unpublished data). However, abrogation of p53 activity may produce more dramatic phenotypes in concert with other viral gene products that affect cell proliferation, like the viral cyclin (see below; and Verschuren *et al.*, 2002).

Another reported activity of LANA is binding to the tumor suppressor Rb (Radkov *et al.*, 2000). While it is unclear what fraction of cellular Rb protein is complexed to LANA, that which is bound is inactivated, as judged by the enhanced activity of E2F reporter genes. LANA can also cooperate in 3T3 cell transformation when coexpressed with activated ras genes, an activity that it shares with other DNA tumor virus oncogenes that inactivate Rb. The significance for PEL of this Rb binding, however, has recently been called into question by the finding that many PEL cell lines display loss of the cyclinD-cdk inhibitor p16INK4a (Platt *et al.*, 2002). Since this protein acts to inhibit cellular cyclind/cdk6 from inactivating Rb, its loss should trigger proliferation – but only in cells with functional Rb. Indeed, PEL cell lines remain sensitive to growth inhibition when p16 INK4a expression is restored by transfection. These results imply that despite expression of LANA, Rb function in PEL cells has not been completely inactivated. The fact that one PEL cell line (BC3) has been identified in which Rb protein is missing altogether (Platt *et al.*, 2002) further sustains this impression. As such, these findings are at variance with what is observed in tumors induced by other DNA tumor viruses that strongly inactivate Rb (Kelley *et al.*, 1995; Wrede *et al.*, 1991); in those cases, lesions in Rb and INK4a loci are very uncommon. The partial inhibition of Rb mediated by LANA may provide a selective advantage early in tumorigenesis, but additional lesions in the pathway appear to be required for full oncogenicity.

Another role for LANA in tumorigenesis stems from recent observations that the protein can interact with GSK-3ß, a kinase that phosphorylates and inactivates ß-catenin by targeting it for ubiquitin-mediated proteolysis (Fujimuro *et al.*, 2003). Binding of GSK-3ß relocates the kinase from the cytosol to the nucleus, allowing cellular accumulation of ß-catenin protein, which can then oligomerize with the transcription factor LEF and turn on a proliferative program of gene expression that includes cyclin D, c- myc, and c-jun. In fact, PEL cells display ß-catenin upregulation, and this can be impaired by siRNAs directed against KSHV LANA (Fujimuro *et al*, 2003). Although the consequences of this for PEL cell growth and survival have not been determined, transient LANA expression in other cell types is associated with enhanced S-phase entry, consistent with the idea that the ß-catenin pathway could be important in KSHV-induced proliferation. And certainly, many other types of cancer are linked to dysregulation of the ß-catenin pathway, including tumors of the colon, esophagus, breast and uterus (Polakis, 2000).

In addition to its effects on ß-catenin/LEF, LANA also can act more directly on the program of host gene expression. Early studies of expression profiling in stably-transfected B cells indicated that many host genes are dysregulated by LANA expression, both positively and negatively (Renne *et al.*, 1996) – though such experiments do not distinguish direct from indirect effects. Other experiments suggest that LANA's principal direct transcriptional activity is repression – for example, LANA will extinguish transcription of reporter genes linked in cis to TR elements containing LANA binding sites; similarly, when LANA is tethered to the DNA binding domain of Gal4, it can repress transcription of GAL4-dependent reporters (Krithivas *et al.*, 2000; Schwam *et al.*, 2000; Garber *et al.*, 2002). This effect is due in part to the recruitment of the mSin3 co-repressor complex via binding to the protein SAP30 (Krithivas *et al.*, 2000). However, it remains unclear how this activity is related – if at all- to oncogenesis (It could, for example, have more to do with the maintenance of viral latency or to the modulation of KSHV chromatin structure.) Similar statements apply to the many other activities described for LANA – such as binding to the nuclear matrix, the bromodomain-containing RING3 protein, me-CpG binding protein 2, heterochromatin protein 1, and various components of the transcription apparatus (Viejo-Borbolla *et al.*, 2003; Platt *et al.*, 1999; Mattsson *et al.*, 2002; Krithivas *et al.*, 2002; Lim *et al.*, 2001, 2003)

v-cyclin

Cotranscribed with LANA from the major latency promoter is a viral homologue of cellular cyclin D. This protein, v-cyclin, is expressed principally from a spliced mRNA from which the upstream LANA gene has been removed (Dittmer *et al.*, 1998; Sarid *et al.*, 1999). V-cyclin is a fully functional cyclin (Chang *et al.*, 1996) in that it can bind to and activate cdk6 (though unlike cellular cyclin D family members, it is much less active on cdk4). The in vitro substrate specificity of v-cyclin/cdk 6 differs

substantially from that of host cyclinD/cdk6; allthough both can phosphorylate Rb, the viral cyclin can also trigger cdk6 phosphrylation of p27, histone H1, Id-2 and cdc25a (Godden-Kent *et al.*, 1997; Li *et al.*, 1997). Forced v-cyclin expression can induce S-phase entry in quiescent 3T3 cells, and also overcome an Rb-mediated growth arrest induced by cdk-inhibitors in cultured cells (Swanton *et al.*, 1997). In fact, the v-cyc/cdk6 complex is less sensitive to inhibition by cdk inhibitors like p27, p21 and p16. For p27, at least, this resistance is further accentuated by the fact that the p27 protein is targeted for degradation by dint of its phosphorylation by v-ccylin/cdk6 (Mann *et al.*, 1999; Ellis *et al.*, 1999).

These activities suggest that v-cyclin might promote cellular proliferation in vivo. However, it has been difficult to document such a role for this gene in viral tumorigenesis in vivo, and the study of gene expression in primary PEL tumors turns up numerous discrepancies with the picture painted by studies of v-cyclin biochemistry. For example, despite the fact that v-cyclin expression destabilizes p27 in cultured cells, PEL tumors routinely display abundant p27 expression (Carbone *et al.*, 2000). And the fact that many PEL tumors delete p16INK4a suggests that despite the action of v-cyclin, Rb function is not fully abrogated in latently infected B-cells; further mutational lesions must accumulate in this pathway for full transformation. What v-cyclin does in the economy of the cell prior to the advent of such mutations remains the province of inference, extrapolation, and analogy.

Compounding the dilemma, we lack many experimental tools that are standardly used to approach such problems in other systems. First, there are no animal models of lymphomagenesis by KSHV in which critical notions about v-cyclin's role could be put to the test – for example, via the study of v-cyclin-deficient mutants. Another impediment has been the difficulty in establishing stable cell lines expressing the protein. This is most likely due to the fact that such cells often undergo apoptosis, particularly if they express elevated levels of cdk6 (Ojala *et al.*, 1999). Such problems are not unexpected: apoptosis often follows overexpression of cellular cyclins, and many viral oncogenes that provoke unscheduled DNA synthesis can similarly induce p53 and trigger programmed cell death. V-cyclin- induced apoptosis is also associated with the inactivation of the antiapoptotic factor bcl2 – which, it turns out, is due to its phosphorylation at the hands of vcyclin/cdk6 (Ojala *et al.*, 2000). It is unclear how this problem is mitigated in vivo, but the low levels of v-cyclin protein accumulation in latency likely represent one viral stratagem for doing so. Alternatively, another viral gene product might nullify this apoptotic effect. In this connection it is of interest that Verschuren *et al.* (2002) have shown that in cultured cells, loss of p53 allowed cells to survive in the presence of elevated levels of v-cyclin. Moreover, when v-cyclin was targeted to the B cell lineage in transgenic mice, lymphomas were observed only when the animals were also p53 -/-. This raises the possibility that the functional inactivation of p53 by LANA expression in KSHV latency (or by v-IRF3; see below) might similarly unmask the oncogenic potential of v-cyclin.

v-FLIP

The third protein encoded by the major latency locus is a viral homologue of the FLICE inhibitory protein, v-FLIP. Transcribed from the same promoter as LANA and v-cyclin, vFLIP is the downstream gene in the spliced, bicstronic mRNA from which v-cyclin is expressed. The failure of attempts to identify moncistronic vFLIP transcripts led to the recognition that an IRES element is embedded within v-cyclin coding sequences (Bieleski and Talbot, 2001; Grundhoff and Ganem, 2001; Low *et al.*, 2001). This implies that both the v-cyclin and LANA mRNAs could template vFLIP translation, though the greater abundance of cyclin mRNA makes it the predominant vFLIP message.

FLIPs as a class are thought to bind adaptor proteins (TRADD and FADD) of the Fas/TNFR signaling pathway via their death effector domains (DEDs) (Thome *et al.*, 1997). This binding impairs the recruitment and activation of caspase 8, leading to blockade of a caspase activation cascade that results in programmed cell death; procaspase 8 can also be directly bound and inhibited. Several other herpesviruses encode v-FLIPs that have been shown to employ this mechanism (Thome *et al.*, 1997). Some papers report that KSHV v-FLIP can function in this fashion (Djerbi *et al.*, 1999, Belanger *et al.*, 2001). However, several laboratories have failed to confirm these findings, and other reports suggest that KSHV v-FLIP can also block cell death via induction of the anti-apoptotic transcription factor NFkB (Chaudhary *et al.*, 1999). vFLIP can be detected in PEL cells complexed wiith NEMO (or IKKγ) (Liu *et al.*, 2002; Field *et al.*, 2003). This complex activates IKK, leading to phosphorylation of IkB and release of active NFkB. These findings are particularly provocative in view of the observations that (i) PEL cells display high levels of NFkB activity, inhibition of which triggers enhanced cell death (Keller *et al.*, 2000); and (ii) that siRNA-mediated inhibition of v-FLIP in PEL cell lines provokes apoptosis (Guasparri *et al.*, 2004; Godfrey *et al.*, 2005). It thus appears that the major role of v-FLIP in PEL latency in the activation of an anti-apoptotic program, principally through upregulation of NFkB activity. However, KSHV vFLIP also binds to TRAF2

and RIP upstream of IKK, and TRAF binding also results in JNK activation (An *et al.*, 2003) – the roles of these activation events in cell proliferation and survival are not yet understood. Very recently, it has been reported that forced overexpression of KSHV vFLIP in rodent fibroblasts results in classical transformation – growth in soft agar and tumorigenesis in nude mice (Sun *et al.*, 2003). These studies raise the possibility that deregulated vFLIP could contribute to an active proliferative program rather than simply extending cell survival. However, such inferences must be made with caution, since (i) the levels of vFLIP in PEL cells are likely much lower than those achieved in these studies, and (ii) the transforming function of vFLIP occurs in the context of antecedent immortalization, and in cells of heterologous species and cell type.

Finally, other studies (Brown *et al.*, 2003) suggest an additional role for NFkB activation by v-FLIP in KSHV biology. Activation of NFkB inhibits lytic cycle gene expression, and inhibition of NFkB activation promotes lytic reactivation. This implies that apart from its role in cell survival, V-FLIP-mediated NfkB activation may be important for maintaining stable KSHV latency.

K10.5/v-IRF3/LANA2

The KSHV genome harbors at least four genes that encode members of the IRF (*i*nterferon *r*egulatory *f*actor) transcription factor family. IRFs are a family of cellular transcription factors, several members of which (esp. IRF3 and IRF 7) are centrally involved in the transcriptional activation of type I interferons – hence their name (Barnes *et al.*, 2002). The KSHV IRF homologues (generically termed v-IRFs) are clustered together in one region of the viral chromosome, suggesting that they arose by duplication of one or more ancestral gene(s) presumably derived from the host genome. Most of the vIRFs are lytic proteins, but one, v-IRF3 (encoded by ORF K10.5), encodes a nuclear protein expressed in latency (Rivas *et al.*, 2001; Lubyova and Pitha, 2000). (For this reason it has also been dubbed LANA-2.) Unlike the LANA/v-cyclin/v-FLIP locus, the vIRF3 gene is not expressed in all latently infected cell types in vivo. Its products are found primarily in latently infected B cells, including PEL cells and cells from MCD, but not in KS spindle cells (Rivas *et al.*, 2001). Like several of its sister vIRFs, vIRF3 expression in mouse fibroblasts inhibits the IRF3/7-mediated induction of the IFN-A4 promoter and impairs the ability of these cells to support IFN induction in response to RNA virus infection – suggesting that vIRF3 can function like a dominant-negative (DN) IRF mutant (Lubyova and Pitha, 2000). However, more recent results in human cells have disputed this simple conclusion, suggesting instead that vIRF3 can actually enhance the binding of IRFs 3 and 7 to the IFN promoters and upregulate IFN release (Lubyova *et al.*, 2003); if so, it is difficult to understand the biological rationale for such an activity, especially since IFNs can be growth-inhibitory in some settings. Easier to relate to the biology of KSHV latency is the observation that vIRF3 protein can also bind and inactivate p53 (Rivas *et al.*, 2001); this activity would be expected to contribute to lifespan prolongation in KSHV-infected B cells, particularly in the context of v-cyclin expression (Verschuren *et al.*, 2002). All of these notions, however, remain to be explicitly tested.

Kaposin

The kaposin locus was initially identified in a screen for genes that are transcibed in uninduced PEL cell cultures (Zhong *et al.*, 1996). In situ hybridization using probes for kaposin mRNA revealed that it is expressed in both uninduced BCBL-1 cells (at a low level) and in variable levels in KS spindle cells lacking markers of lytic reactivation (Staskus *et al.*, 1997; Sturzl *et al.*, 1997). Thus, kaposin transcripts behave as latent mRNAs that, like the LANA/vcyclin/vFLIP mRNAs, can be expressed in both lymphoid and endothelial cells. Recent work from several laboratories has mapped these latent mRNAs and identified their promoter (Li *et al.*, 2002; Pearce *et al.*, 2005; Cai and Cullen, 2006). A second promoter in the kaposin locus is strongly induced during lytic replication (Sadler *et al.*, 1999), owing to the presence of at least one high-affinity binding site for the lytic switch protein RTA (Chang *et al.*, 2002; Song *et al.*, 2003)

Kaposin mRNA encodes at least three proteins via differential initiation of translation (Fig. 56.3(a)) (Sadler *et al.*, 1999). The 3 end of kaposin mRNA bears the coding sequences of ORFK12, initiation at whose AUG directs the synthesis of a 60aa hydrophobic polypeptide known as kaposin A. The protein is found on both the cell surface and on intracellular membranes (Tomkowicz *et al.*, 2002). Interest in this protein was heightened when it was shown that its overexpression in immortalized rodent fibroblasts can trigger morphologic transformation in vitro and tumorigenesis in vivo (Muralidhar *et al.*, 1998). Subsequently it was found that kaposin A can bind cytohesin - 1, a guanine nucleotide exchange factor (GEF) for ARF GTPases and a regulator of integrin-mediated cell adhesion. Binding of kaposin A to cytohesin-1 stimulates GTP binding to ARF1, a process that is implicated in transformation since the transformed phenotype can be reverted by expression of a cytohesin-1 mutant deficient in guanine nucleotide exchange (Kliche *et al.*, 2001). Kaposin A has growth deregulatory potential,

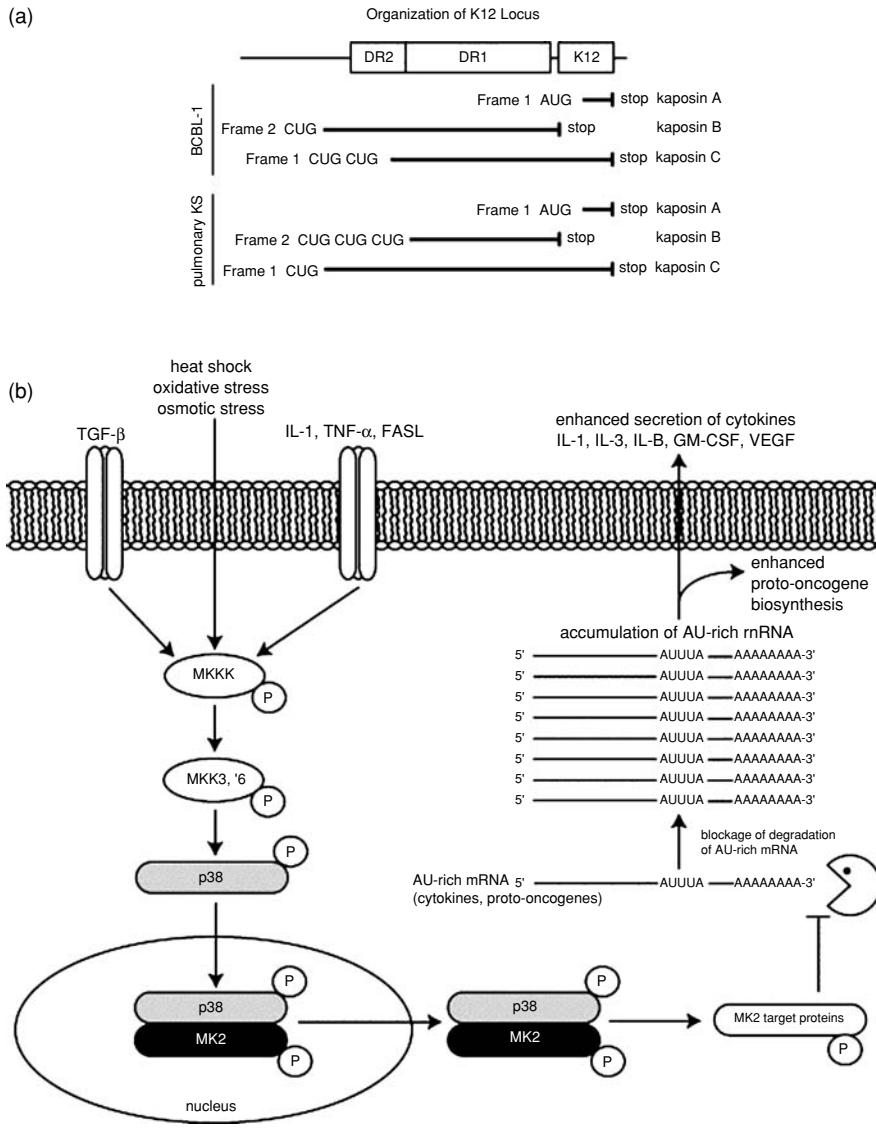

Fig. 56.3. (a) The kaposin locus. Top line, sequence organization of the gene,showing open reading frame (IRF) K12 and the upstream series of tandem direct repeats (DRs) of 23 GC- rich bp. The locus comprises two sets of such Drs(DR1 and DR2) which differ in sequence from each other. Kaposins B and C are derived from translation of the repeats via initiation from CUG codons in different reading frames; kaposin B is encoded solely by the repeats, while kaposin C extends into ORF K12. Sequence differences between different isolates of KSHV result in the production of kaposins B and C from CUGs located at different locations in the locus; shown here to illustrate this is the coding arrangement in KSHV cloned from BCBL-1PEL cells (top), and a primary KS tumor from lung tissue(bottom). (b) Schematic illustration of the p38/MK2 pathway. Activation of p38 via inflammatory or others stresses leads to its nuclear import, where it binds and phoyphorylates MK2 can phosphorylate some nuclear transcription factors. Active MK2-p38 complexes can also be exported from the nucleus to the cytosol, where MK2-p38 complexes can also be exported from the nucleus to the cytosol, where MK@ can phosphorylate additional targets involved in stabilizaton of transcripts bearing AU-rich elements (AREs). Such AREs are typically found in cytokine mRNAs. Kaposin B binds and activates MK2 in a fashion that is independent of upstream inflammatory or oxidative stresses.

especially in cells that have already undergone immortalization. But determination of its role in authentic KSHV tumorigenesis will require examination of its effects in primary cells of lymphoid and endothelial lineage. Such tests should be possible in transgenic mice, though none has yet been reported.

Immediately 5[1] to the K12 open reading frame is a series of tandemly repeated 23-nt GC-rich elements of two families, termed DR (*d*irect *r*epeat) 1 and DR2. Because this region lacks AUG codons, it was initially assumed to be noncoding. However, multiple proteins are produced from this region by initiation at variant CUG codons. (Sadler *et al.*, 1999). One of these, Kaposin B, is encoded predominantly by the repeat sequences; another, kaposin C, expresses the repeats fused in frame to ORF K12, producing a membrane-bound isoform of kaposin B. The functions of kaposins B and C have long been mysterious, but recent findings indicate that they likely function as adapter proteins in signal transduction. Specifically, they bind and activate a kinase, MK2, that is normally a target of p38 phosphorylation (McCormick and Ganem, 2005; Fig 56.3(b) summarizes the key features of the p38-MK2 pathway). The activation mechanism is complex, but appears to require p38, suggesting that kaposin B either increases the sensitivity of MK2 to p38, or decreases the accessibility of p38-phophorylated MK2 to phosphatase action. One result of this activation is the stabilization of mRNAs bearing AU-rich elements in their 3 UTRs (See Fig 56.3(b))– an important result, since such elements are found primarily in the highly regulated transcripts of key cytokines (e.g. IL1,3,4,6, TNF, GM-CSF), growth factors (VEGF) and oncogenes (c-myc). PEL cells are known to produce abundant cytokines (notably IL6, vIL6 and IL10) and many PEL lines are dependent upon one or more of these factors for cell growth or survival in vitro (Aoki *et al.*, 2000; Jones *et al.*, 1999). Cytokines and growth factors also play important roles in KS pathogenesis (see below), and it is likely that kaposin contributes to their production in this disease as well. It is important to point out, however, that post-transcriptional regulation by kaposin is not likely to be the sole modulator of cytokine and growth factor expression in KSHV infection – many of these genes may be controlled transcriptionally as well, by both viral and host factors (see, for example, An *et al.*, 2002).

Latent KSHV miRNAs

Recently it has become clear that in addition to the aforementioned coding RNAs, the latency program of KSHV also generates up to 12 micro RNAs (miRNAs) (Pfeffer *et al.*, 2005; Cai *et al.*, 2005; Samols *et al.*, 2005). These miRNAs are derived primarily from the latent kaposin mRNAs, with all but two emanating from intronic regions of the transcripts (Cai and Ciullen, 2006). (The two encoded in the body of the kaposin mRNA could arise from either latent or lytic kaposin RNAs, but they are only weakly induced during lytic replication.) The function(s) of these miRNAs are not known.

Other KSHV genes and PEL

There is general agreement that the aforementioned genes are *bona fide* products of viral latency. However, there is one viral gene with a likely role in PEL that has not been definitively classified as a latent gene – that for the viral homologue of IL6 (v-IL6). In most cultured PEL cells, this gene is very weakly expressed in the absence of chemical inducers of lytic reactivation, and is strongly upregulated by the latter. This has led most to characterize it as a lytic gene (Nicholas *et al.*, 1997; Staskus *et al.*, 1999; Katano *et al.*, 2000); the low-level expression in the absence of inducers has been ascribed to spontaneous lytic reactivation. However, Parravicini *et al.* (2000) observed by immunohistochemistry on PEL biopsies that a small number of cells in the tumor expressed vIL6 in the absence of another marker of lytic reactivation (vIRF1). They have proposed on this basis that some primary PEL tumor cells can express v-IL6 in the absence of lytic reactivation, though this appears not to be the case for most latently infected PEL cells. Interestingly, when cultured PEL are exposed to IFN-γ, they can express vIL6 without activating any other viral gene (Chatterjee *et al.*, 2002); this lends credence to the idea that the gene can sometimes be expressed outside of the lytic cycle. Whether vIL6 is expressed from a small number of lytically infected cells or is coming from an alternative transcriptional program, there is evidence that its action is important in PEL biology. All PEL cells express the gp130 subunit that allows response to vIL6 (Molden *et al.*, 1997), and in cultured PEL lines, growth can be partially inhibited by anti-vIL6 (but not anti-IL6) antibodies (Jones *et al.*, 1999). (More dramatic inhibition is obtained when both human IL10 and vIL6 are inhibited; Jones *et al.*, 1999.) These results suggest that despite their seeming autonomy, PEL cells do respond to autocrine and paracrine signals, and that these signals can emanate from the host as well as the viral genome.

Multicentric Castleman's disease (MCD)

Castleman's disease is a polyclonal lymphoproliferative lesion that occurs in two histologic types. The first, or

"plasma cell variant" is characterized by extensive plasma cell proliferation but preservation of the nodal architecture. The second or "hyaline-vascular" type, displays prominent, abnormal germinal centers and marked, aberrant neoangiogenesis. Clinically, Castleman's disease occurs in two forms, a localized form involving only a single node or node cluster; this form is unlinked to KSHV and can be treated with local excision of the involved tissue. The second form, multicentric Castleman's disease (MCD), is an aggressive systemic illness characterized by fever, weight loss, splenomegaly and diffuse lymphadenopathy. MCD occurs at increased frequency in AIDS patients, and in this context is nearly always linked to KSHV infection (Soulier *et al.*, 1995); histologically, these cases are usually of the plasma cell type. However, MCD does occur rarely in HIV-negative subjects; in this context, only 40–50% of cases show KSHV DNA in the lesions (Soulier *et al.*, 1995; Parravicini *et al.*, 1997). Although polyclonal (Du *et al.*, 2001), MCD is an aggressive disease that is often treated with chemotherapeutic drugs just like a lymphoma. In fact, microscopic foci of clonal plasmablastic lymphoma have been reported to arise within foci of MCD (Dupin *et al.*, 2000; Oksenhendler *et al.*, 2002).

The polyclonality of MCD suggests that the process is being driven by paracrine signaling factors to which a large number of B cells can respond. Consistent with this idea, all forms of MCD appear to be associated with the overproduction of IL6, and there are strong reasons to implicate IL6 signaling in disease pathogenesis. First, IL6 is a known inducer of B cell differentiation, proliferation and survival (Horn *et al.*, 2000). Second, IL6 overproduction in mice leads to an MCD-like illness (Brandt *et al.*, 1990). Third, high levels of circulating IL6 correlate with MCD exacerbations (Oksenhendler *et al.*, 2000). And fourth, administration of neutralizing IL6 mAbs to patients with MCD reverses their fever and other systemic symptoms and ameliorates their splenomegaly (Beck *et al.*, 1994), although this finding has been tested primarily in HIV-negative MCD patients, not all of whom are KSHV-positive.

KSHV infection is localized to the B cells of the "mantle zones" surrounding the germinal centers of MCD (Dupin *et al.*, 1999; Parravicini *et al.*, 2000; Katano *et al.*, 2000). Despite the fact that MCD is a polyclonal lesion, all KSHV-positive cells in the lesion bear lambda light chains, usually on IgM (Du *et al.*, 2001) – the origin of this peculiar finding remains unclear. Many of these cells are latently infected, as judged by the presence of LANA staining – from 10–50% of mantle zone cells (typically around 30%) have been reported to be LANA-positive in various series (Dupin *et al.*, 1999; Parravicini *et al.*, 2000; Katano *et al.*, 2000). In addition to KSHV latency, however, MCD also displays abundant lytic infection. From 5–25% of LANA-positive cells in the mantle zone stain for lytic markers – many more than in PEL or KS (Staskus *et al.*, 1999; Katano *et al.*, 2000, Parravicini *et al.*, 2000). These lytically infected cells stain prominently for v-IL6 – an important finding given the suspected role for IL6 signaling in this disease. (Although IL6 and vIL6 differ in their requirement for IL6Rα, both signal via gp130 and thus induce nearly identical signals in recipient cells (Nicholas 2003).) Clinical evidence also links MCD to lytic KSHV replication. MCD typically occurs in patients with high KSHV loads in the PBMC compartment, a manifestation of extensive lytic growth (Oksenhendler *et al.*, 2000, Grandadam *et al.*, 1997, Boivin *et al.*, 2002). In fact, an MCD-like syndrome has even been seen in primary KSHV infection (Oksenhendler *et al.*, 1998), where lytic infection is typically predominant. (It should be emphasized, however, that most primary infections with KSHV are subclinical and do not engender MCD).

The discovery that vIL6 production is prominent in the expression program of MCD is satisfying, especially given its ability to signal along pathways shared by human IL6 (Nicholas, 2003). Nonetheless, it is clear that human IL6 continues to be overproduced even in KSHV-associated MCD (Oksenhendler *et al.*, 2000), and it is difficult to judge the relative contributions of each of these two proteins to the disease state. Most experts assume that vIL6 is predominant, but there is little direct evidence on either side, and it seems likely that both proteins contribute to the disease. The source of human IL6 in KSHV-linked cases is now starting to become clear. One pathologic study shows that h-IL6-positive cells are found mainly within the follicular centers (Parravicini *et al.*, 1997) – a place with few KSHV-positive cells. This suggests that one source is reactive, uninfected B lymphocytes. However, some mantle-zone cells in that study were also positive, and it has recently been found that lytically infected cells produce high levels of cellular IL6 as well as v-IL6 (Glaunsinger and Ganem, 2004b). Given the extensive amount of lytic replication in MCD, and the correlation of this with both disease progression and circulating human IL6 levels, it is reasonable to suspect that these cells are also contributing to the raised steady-state levels of h-IL6.

Kaposi's sarcoma

The choice of the word sarcoma in the naming of KS is unfortunate, for KS resembles classical sarcomas very little, both in terms of its histology and clinical behavior.

Unlike most classical cancers, which arise as a clonal outgrowth of a single cell type, KS lesions are histologically very complex (Regezi *et al.*, 1993a,b). The main proliferating element is the so-called *spindle cell*, so named after its spindle-like shape. Marker studies indicate that this cell is in the endothelial lineage, though there has been controversy over its exact position in this lineage (Herndier and Ganem, 2001). Some evidence favors it being derived from lymphatic rather than blood vessel endothelium (Beckstead *et al.*, 1985), based upon the expression of markers characteristic of lymphatic endothelium such as VEGF-R3, VEGF-C and podoplanin (Weninger *et al.*, 1999; Skobe *et al.*, 1999; Marchio *et al.*, 1999). However, this conclusion is still inferential, and spindle cells within any given lesion can display variability in the expression of markers (Regezi *et al.*, 1993a,b). Moreover, recent studies show that KSHV infection of vascular epithelial cells can reprogram them to express lymphatic markers (Wang *et al.*, 2004; Hong *et al.*, 2004; Carroll *et al.*, 2004). Irrespective of their precise origin, spindle cells clearly represent the main proliferative element in KS. However, they are not the sole element – all KS lesions also contain (i) variable (but substantial) numbers of infiltrating monocytes, T-cells and plasma cells; and (ii) a profusion of aberrant, slit-like neovascular spaces. These neovascular channels, the most distinctive histologic signature of KS, are only partially lined with KSHV-positive cells (Dupin *et al.*, 1999), lack pericyte or smooth muscle accompaniment, and are prone to leakage and rupture. As a result, KS lesions also often display edema and hemorrhage, giving them a grossly purplish or bruise-like appearance. It is thus useful to think of KS as subsuming three formally separable (but probably interdependent) processes – a *proliferative* component (chiefly involving spindle cells), an *inflammatory* component and an *angiogenic* component. As we shall see, KSHV may make different genetic contributions to each of these components of the disease.

KS can occur in several tissues, but is most commonly localized to the skin, especially in the lower extremities in classical, HIV-negative forms of the disease. In the skin, it involves the dermis, with sparing of the overlying epidermis (Parravicini *et al.*, 2000). Cutaneous involvement, while potentially disfiguring, is not life-threatening. Dermal KS begins as a so-called *patch* lesion, in which spindle cells are clearly demonstrable but are not necessarily the dominant element of the lesion. Inflammatory cells and neovascular elements are prominent at this stage, which can somewhat resemble granulation tissue. Subsequently, the accumulation of spindle cells becomes more apparent, as the lesion progresses through a plaque-like stage to the *nodular* stage, in which masses of spindle cells generate macroscopically visible nodules or masses.

As noted above, the clinical behavior of KS in immunocompetent adult hosts is very indolent- cutaneous KS is typically a disease which people die *with* but not *of*. In fact, spontaneous remissions of classical KS, while not common, are well-documented (However, in immunocompromised hosts – and in young children in KSHV-endemic regions – KS can be more aggressive, involving lymphoreticular structures, the GI tract and the lung.) This indolent behavior suggests that KS is a proliferative state that is on the cusp between the benign and the malignant. Consistent with this inference, analysis of KS clonality has shown that many KS lesions are oligo- or polyclonal (Judde *et al.*, 2000; Gill *et al.*, 1998; Delabesse *et al.*, 1997). While this conclusion has been disputed by some (Rabkin *et al.*, 1997), their own analyses showed evidence of non-clonality in numerous tumors. The heated controversy surrounding KS clonality is misplaced. The pathogenetically important finding is that many macroscopic KS lesions have evidence of oligo- or polyclonality – not that *all* of them do. In any polyclonal process, it is expected that, in some cases, some clones will fare better than others. The instructive feature of the studies of clonality in KS is how different they are from similar studies of classical cancers like breast or colon cancer – in which exceptions to monoclonality are almost never found.

The cell biology of the spindle cell also supports the view that KS is at a (somewhat indistinct) border between hyperplasia and neoplasia. KS spindle cells, unlike many tumor cells, remain highly dependent upon exogenous growth signals when cultured in vitro. Gallo, Ensoli and their colleagues were the first to find methods to reproducibly grow KS spindle cells in culture, and found them to be dependent upon the cytokine-rich conditioned medium of activated T-cells (Caveat: after in vitro culture, the cells produced in this fashion generally lose the viral genome; see below.) (Ensoli *et al.*, 1989, 2001; Miles *et al.*, 1990; Ensoli and Sturzl, 1998). Nor do they display genetic instability, another hallmark of traditional cancers: KS spindle cells are generally diploid, and classical KS lesions do not display microsatellite instability, though some advanced AIDS-KS lesions do (Bedi *et al.*, 1995). Spindle cells also lack classical findings associated with transformation – they do not form foci, grow in soft agar or form tumors in nude mice. However, an interesting phenotype is observed when the cells are injected subcutaneously in nude mice (Salahuddin *et al.*, 1988). They do not grow, but survive for a brief interval – during which slit-like new vessels *of murine origin* appear in the surrounding tissue. When the human implant involutes, these vessels likewise disappear. This suggests a model for KS that involves a complicated paracrine minuet between several partners. In this model,

spindle cells require growth factors from their microenvironment (perhaps from infiltrating inflammatory cells) for their proliferation, but also seem to produce angiogenic (and proinflammatory?) substances to recruit the other components of the lesion. In this view, none of the partners in this process is fully autonomous; each depends upon the other (Ensoli and Sturzl, 1998). If correct, this is a very different biology than most classical cancers – so it should not surprise us if the viral contributions to its pathogenesis turn out to differ from those of classical DNA tumor viruses.

KSHV and KS: the big picture

The epidemiology of KSHV strongly points to a critical role for this infection in KS pathogenesis – KSHV DNA is always found in KS tumors, irrespective of whether they derive from classical or AIDS-related KS (Chang *et al.*, 1994; Moore and Chang 1995; Huang *et al.*, 1995; Schulz 1999). At the population level, KSHV infection tracks strongly with KS risk – the prevalence of infection is high in groups in which KS is frequent, and low in those in which it is rare (Kedes *et al.*, 1996; Gao *et al.*, 1996). This statement applies both to risk groups within the USA and to populations around the globe (Schulz, 1999). At the level of individual HIV-infected patients, infection with KSHV preceeds the onset of KS (Moore *et al.*, 1996a,b) and predicts an elevated risk of KS tumorigenesis (Martin *et al.*, 1998).These data, which in the aggregate derive from the study of thousands of subjects, strongly indicate that KSHV infection is the *sine qua non* of KS – without it, clinical KS does not occur. But to say it is necessary for KS development is not to say it is sufficient – indeed, there is strong evidence that it is not. First of all, 2%–7% of the US population has serologic evidence of infection (Schulz, 1999), yet most of these individuals have no measurable risk of developing KS. This indicates that cofactors are clearly required for tumorigenesis. In AIDS-related KS, that cofactor is clearly HIV infection – a fact that is strongly supported by (i) the dramatic reduction in KS incidence that has accompanied reduction of HIV viremia via antiretroviral therapy (Portsmouth *et al.*, 2003; Gates and Kaplan 2002); and (ii) the remission of clinical KS in patients treated with HAART alone (Lebbe *et al.*, 1998; Cattelan *et al.*, 2001; Gill *et al.*, 2002). HIV-1 infection predisposes to KS much more strongly than does HIV-2 infection (Ariyoshi *et al.*, 1998). How HIV infection promotes KS development is still actively debated. Several in vitro studies have suggested that HIV infection can augment KSHV replication in both cell-autonomous and paracrine fashions (Harrington *et al.*, 1997; Varthavaki *et al.*, 1999, 2002; Huang *et al.*, 2001). Secreted HIV tat protein has also been proposed as a growth factor for KS spindle cells, again on the basis of experiments in cultured cells (for review see Barillari and Ensoli, 2002). However, the in vivo importance of these observations remains to be proven. Cell-autonomous mechanisms of KSHV activation, for example, require that cells be dually infected with both HIV and KSHV – which is exceedingly rare in vivo (Staskus *et al.*, 1997). (This caveat does not apply to paracrine mechanisms of activation). The fact that KS is strongly augmented by therapeutic immunosuppression (e.g., with cyclosporin) makes it likely that T cell depletion by HIV is also centrally involved in the amplification of KS risk, e.g., due to the inability to contain KSHV replication.

The identity of the cofactor(s) in classical KS remain unknown. Their characterization is the last remaining Great Unsolved Problem in the human biology of KS, Unfortunately, though – and in marked contrast to AIDS-KS (Beral *et al.*, 1990) – the epidemiology of classical KS has not proffered strong clues to their identity. There are hints, however, that host genetic factors may play a role. Such hints derive from the identification of populations that have a low risk of classical KS despite a high prevalence of KSHV infection. In one such group (Ethiopians), even after the superventionof AIDS KS developed at a 12-fold lower rate than in other AIDS-afflicted groups (Grossman *et al.*, 2002). In such populations whatever host factors are modulating KS risk, they must operate post the acquisition of KSHV.

KSHV gene expression in KS

As noted above, KSHV DNA is present in all KS tumors, irrespective of clinical type or disease stage. Within the tumor, KSHV specifically targets the spindle cell compartment (Boshoff *et al.*, 1995; Staskus *et al.*, 1997; Dupin *et al.*, 1999), with little or no infection of other cell types. (One report suggests that monocytes in the lesions may be infected (Blasig *et al.*, 1997), but this has not been confirmed in other studies (Parravicini *et al.*, 2000; Staskus *et al.*, 1997.) Using known latency genes (as defined by their pattern of expression in PEL cell lines) as probes in *in situ* hybridization studies, it was shown that most KS spindle cells are latently infected; however, a small subpopulation (1%–2%) express markers of lytic infection (Staskus *et al.*, 1997; Dupin *et al.*, 1999; Sturzl *et al.*, 1997, Katano *et al.*, 2000). Several groups have enumerated the latency genes expressed in KS spindle cells in vivo, using immunohistochemistry or *in situ* hybridization on KS biopsies. In general, all of the genes discussed previously in PEL latency are expressed in latently infected spindle cells, save for v-IRF3 (LANA 2) (Rivas *et al.*, 2001). Careful studies (Katano *et al.*,

2000; Parravicini *et al.*, 2000) also show that, in KS, v-IL6 is produced only in the minor subset of cells that is truly in the lytic cycle – here, there is no suggestion that KS cells can express this gene outside of the lytic cycle.

The discovery that most spindle cells display latent infection is in accord with herpesviral dogma that the latency program drives tumor formation. Consistent with this, examination of early (patch) lesions of KS shows that only 10–30% of spindle-like cells are LANA-positive (Dupin *et al.*, 1999), while in later lesions virtually all such cells are latently infected (Staskus *et al.*, 1997; Dupin *et al.*, 1999). This clearly implies that latently infected cells have a growth (or survival) advantage in vivo. And certainly, many of the in vitro properties of the latency genes are commensurate with this inference. Examples include: the inhibition of p53 and the partial abrogation of Rb by LANA, the induction of NFkB by v-FLIP, the stimulation of cdk6 by v-cyclin, the potentiation of growth deregulation by kaposin A and the upregulation of ß-catenin by LANA. All of these activites could contribute to endothelial cell survival and proliferation, and, as noted earlier, LANA expression can indeed prolong the lifespan of primary endothelial cells in culture (Watanabe *et al.*, 2003). But since most of these activities were discovered in experiments involving overexpression of individual viral proteins in heterologous cell types that were already immortalized, their contributions to the in vivo biology of KS remain to be defined.

Another experimental perspective on this issue comes from examination of cells infected with authentic KSHV. When KSHV virions are applied to most established cell lines, a latent infection results that appears to have no phenotypic consequences (Viera *et al.*, 2001; Lagunoff *et al.*, 2002; Bechtel *et al.*, 2003). However, when KSHV is used to infect *primary endothelial cells*, the situation is dramatically different. In the early days of the culture, there is dramatic lytic replication and fairly efficient spread of infection through the monolayer (Ciufo *et al.*, 2001; Gao *et al.*, 2003; C. Grossman and DG, unpublished data). By 10–14 days, the entire culture is LANA –positive, with most cells being latently infected. These cells also display a dramatic elongation to a spindle morphology (Fig. 56.4) that is remarkably similar to that seen in KS tumors in vivo (Fig. 56.4). Importantly, however, in most experimenters' hands the cells are not immortalized (Gao *et al.*, 2003, Tang *et al.*, 2003; Grossman *et al.*, 2006), though there is one dissenting report (Flore *et al.*, 1998). While they may undergo some prolongation of lifespan, KSHV-infected endothelial cells are not capable of indefinite proliferation – at least not in conventional culture media. The cells fail to grow in soft agar, and no reports of tumorigenesis by KSHV-bearing cells in nude mice have yet appeared. While these results could simply reflect the inadequacy of current culture conditions, they certainly suggest that, at a minimum, the KSHV latency program is much less potent than that of EBV in cell immortalization. This, in turn, raises questions as to whether this more subtle latency phenotype is sufficient to drive KS pathogenesis all by itself.

In fact, independent evidence from clinical studies suggests that lytic replication as well as latency also plays a pivotal role in KS development. Martin *et al.* (1999) showed that in patients with advanced AIDS, addition of parenteral ganciclovir – a drug that blocks lytic but not latent KSHV infection – resulted in a prompt and dramatic decline in the incidence of new KS tumors. This is a powerful and interesting result because most of these patients have carried HIV and KSHV for many years – therefore, the impact of GCV is not likely to be due simply to the reduction of early dissemination to target cells. Rather, it appears that ongoing lytic replication is continuously necessary throughout the entire natural history of KSHV infection in order to support lesion formation. Consistent with this, patients with KS typically have high levels of KSHV DNA in the circulation (Whitby *et al.*, 1995; Campbell *et al.*, 2000; Quinlivan *et al.*, 2002; Cannon *et al.*, 2003a,b, Engels *et al.*, 2003) – just as do patients with MCD, another disease in which lytic KSHV replication is thought to play an important role.

How might lytic replication contribute to KS tumor development? Here I outline three non-exclusive possibilities. The first derives from the observation that latent KSHV infection is not immortalizing. If spindle cells cannot grow indefinitely, then in order for a tumor mass to expand, KSHV-positive cells that die must be replaced by new latently infected cells. The most obvious source of such cells would be from *de novo* infection of endothelia with virus produced by lytic replication. The second model derives from recent observations of Grundhoff and Ganem (2004), who showed that when cells newly latently infected with KSHV are placed under conditions of rapid proliferation, they rapidly segregate the viral genome. With continued passage, at least in some lines, rare subpopulations can emerge in which latent infection has been stabilized. In such cells, the KSHV genome can be maintained indefinitely as an autonomous episomal replicon – just as it is in PEL cells in vivo. Examination of these rare, stably latent subclones reveals that *cis*-acting, epigenetic changes in the viral chromosome are responsible for stabilization (though the biochemical nature of these epigenetic changes is still unknown). Two lines of evidence support the relevance of these observations to the in vivo situation. First, exactly the same phenomena are observed in primary endothelial cells, which reproduce the main features of KS spindle cell biology (Grundhoff and Ganem, 2004). More importantly,

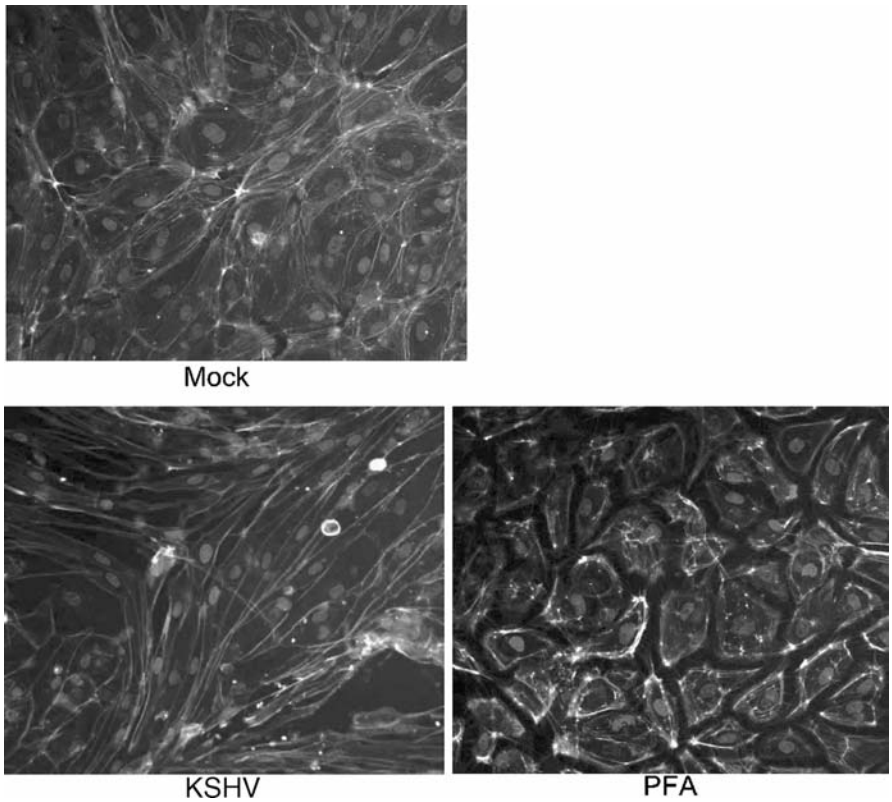

Fig. 56.4. KSHV infection of culture primary endothelial cells results in morphologic changes resembling spindling in vivo. Primary blood endothelial cells were mock infected(top) or infected with KSHV (botttom left); three weeks later, following spread of virus through the culture, the culture was 100% latently infected. Cells were then stained with phalloidin; note extensive spindling of the infected culture.Bottom right: cells were infected with KSHV and immediately carried in Phosphonofomate (PFA), an inhibitor of KSHV replication that blocks spread throught the culture; this blocks widespread establishment of latency and spindling.

when infected KS spindle cells are explanted directly from clinical specimens and placed under conditions favoring their proliferation, they too rapidly – and invariably – segregate their viral episomes (Flamand *et al.*, 1996; Aluigi *et al.*, 1996; Dictor *et al.*, 1996). This instability may be one explanation for the relative clinical indolence of KS, since cells that proliferate aggressively will likely lose their latent infection – and with it their virally endowed growth advantage. Second, it offers another reason why lytic replication is continuously required for KS development – namely, to restore latent infection to spindle cells that have lost their KSHV genomes (or recruit other cells to latency to replace those lost from the latent pool via episome segregation).

The third manner in which lytic KSHV replication may contribute to KS pathogenesis harkens back to the fact that KS is an amalgam of three processes: proliferation, inflammation and angiogensis. Since lytically infected cells die, they cannot contribute importantly to the proliferative component of the disease. However, if lytically infected cells produce paracrine signaling molecules, they could promote both the inflammatory and angiogenic components of the lesion. In fact, examination of the KSHV genome (Russo *et al.*, 1996; Nicholas *et al.*, 1997; Nicholas, 2003) reveals numerous viral genes whose products are secreted signaling molecules – many of which are homoloues of cellular cytokines or chemokines. And interestingly, the majority of these are expressed in the lytic cycle – examples include the three viral CC chemokines and v-IL6. Several of the viral chemokines have been shown to be chemotactic for both Th2 cells (Sozzani *et al.*, 1998; Stine *et al.*, 2000) and endothelial cells (Haque *et al.*, 2001). The former observation suggests that these chemokines may blunt antiviral (and anti-tumor) Th1 responses by favoring Th2 polarization of the local antiviral response – consistent with this, one of these (vCCL2) was shown to extend the survival of cardiac allografts in rodents, in part by impairing CTL infiltration (DeBruyne *et al.*, 2000). The activity on endothelial chemotaxis raises the possibility that KS lesions may be able

to recruit more endothelial targets for infection. Endothelial chemotaxis may also relate to observations that application of several viral chemokines to chick chorioallantoic membranes stimulates angiogensis (Boshoff *et al.*, 1997; Stine *et al.*, 2000), though it is also possible that angiogenesis in these models is due to substances released from inflammatory cells recruited by the chemokines). Another lytic protein, v-IL6, in addition to its known roles in B-cell survival, differentiation and proliferation, has also been suggested to affect local angiogenesis (Aoki *et al.*, 1999), perhaps via induction of VEGF production. Recent studies also indicate that vIL6 signaling can block STAT2 phosphorylation by type I IFN signaling, inhibiting both their growth-arresting potential and also their antiviral action (Chatterjee *et al.*, 2002).

Another viral signaling molecule about which much has been written is the viral GPCR homologue (v-GPCR), a member of the chemokine receptor subfamily of the seven transmembrane G protein coupled receptors (Cesarman *et al.*, 1996). Expression of this protein also is restricted to the lytic cycle (Kirshner *et al.*, 1999; Chiou *et al.*, 2002). The protein displays constitutive signaling activity when expressed in the absence of known ligands, though its activity can be further upregulated by chemokines like GRO-α (Arvanitakis *et al.*, 1997; Gershengorn *et al.*, 1998). The resulting singaling activates the MAPK, PI3-kinase and p38 pathways (Sodhi *et al.*, 2000; Smit *et al.*, 2002), as well as activating NFkB activity (Schwarz and Murphy, 2001), and at least in 3T3 cells can stimulate cell proliferation (Bais *et al.*, 1998). In keeping with this broad signaling activity, many host genes are upregulated in response to vGPCR expression in transfected cells (Polson *et al.*, 2002). Among these is VEGF (Bais *et al.*, 1998; Sodhi *et al.*, 2000), a major mediator of angiogenesis and vascular permeability – and a molecule long suspected of a pathogenetic role in KS based on the prominence of neovascularity and edema in affected tissues. In keeping with these in vitro findings, expression of vGPCR in several different cell lineages in transgenic mice produces focal angioproliferative lesions that share some features with KS (Yang *et al.*, 2000; Guo *et al.*, 2003; Montaner *et al.*, 2003). Because this single viral gene product can be linked to both proliferation and angiogenesis, it is seductive to imagine that it is the key player in KS pathogenesis (Montaner *et al.*, 2003).

Several complicating facts, however, must be reckoned with. First, vGPCR is a lytic cycle gene, and there is no evidence that it is expressed in the majority of proliferating, latently infected KS spindle cells (caveat: for a powerful signaling molecule like this, levels below those detectable by in situ hybridization could still be biologically important). If its expression is restricted to lytic infection, then several conclusions follow. First, its cell-autonomous, pro-mitotic activity is likely to be nullified by the direct cytotoxicity resulting from the lytic cycle. Second, it has recently been found that KSHV lytic replication results in a strong block to *de novo* host gene expression, mediated primarily by virus-induced host mRNA degradation (Glaunsinger and Ganem, 2004a,b). Indeed, in lytically infected endothelial cells in culture, very few of the genes upregulated by vGPCR transfection were found to be expressed – although a limited and transient induction of VEGF-C was observed (Glaunsinger and Ganem, 2004b). It is to be emphasized that these results do not negate a role for vGPCR in KS pathogenesis, but they do place important boundary conditions on its potential roles. For vGPCR to play a role in the proliferative component of the tumor, it will have to be shown that the gene can be expressed outside of the traditional lytic cycle – if not in canonical latency, then in response to some extracellular signal or milieu. While this has not yet been observed, it is not without precedent – recall, for example, that in response to IFN-γ, vIL6 can be expressed by KSHV-positive cells in the absence of any other lyic gene expression (Chatterjee *et al.*, 2002). Even if vGPCR is only expressed in lytic replication, it could still contribute to the angiogenic component of KS, since while the block to host expression is strong, it is not absolute–low levels of host transcripts can still be generated very early in infection, before the establishment of the shutoff. But given the attenuation of its ability to induce VEGF and other genes in this context, it would be predicted that substantial lytic replication would be required to generate KS risk. Interestingly, this is exactly what is observed in vivo – patients with KS generally have very high circulating KSHV loads (Whitby *et al.*, 1995; Campbell *et al.*, 2000; Quinlivan *et al.*, 2002; Cannon *et al.*, 2003a,b, Engels *et al.*, 2003).

Conclusions

The pathogenesis of KSHV-related neoplasms reprises many of the themes struck by other models of herpesviral oncogenesis, but also elaborates variations on them and, more importantly, mandates consideration of entirely new ones. Chief among the latter is the apparent dependence of KS (and likely MCD as well) on continued operation of the lytic cycle. If current notions about this are correct, they have an important potential clinical corollary – namely, that treatment with drugs that block lytic KSHV replication would not only prevent KS development but could potentially arrest or even reverse established KS lesions. Putting this notion into practice, however, may not be simple, as we have no accurate idea of the duration for

which such therapy would need to be administered. This would be expected to depend upon the lifespan of latently infected cells, the frequency with which episomes are segregated in vivo, the levels of KSHV production, and the efficiency of *de novo* infection/reinfection – all factors of which we have little quantitative understanding. Therefore, treatment duration will have to be determined empirically, and is almost certain to be many months. This, in turn, will require therapies that are safe, non-toxic and orally bioavailable. Whether currently available drugs meet these criteria is debatable, and less toxic and expensive drugs may well be required. If such can be found, however, it would allow this notion to be put to its definitive test in a clinical trial.

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Part IV

Non-human primate herpesviruses

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Monkey B virus

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Introduction

B virus (Cercopithecine herpesvirus 1, herpesviridae), an alphaherpesvirus endemic in macaque monkeys, has the unique distinction of being the only one of nearly 35 identified non-human primate herpesviruses that is highly pathogenic in humans. B virus has been positively linked with more than two dozen human deaths since the first report describing it in 1933, five of those in the last 12 years, following exposures involving macaques in during acute B virus infection. B virus, unique among the non-human herpesviruses, is included in this volume because it is distinctively neurotropic and neurovirulent in the foreign human host inadvertently exposed by handling macaque monkeys generally used in biomedical research. Untreated B virus infections in humans result in an extremely high mortality rate (~80%) and, consequently, present unique and potentially lethal challenges for individuals handling macaque monkeys or macaque cells and tissues. Infection in humans is associated with breach of primary skin or mucosal defenses and subsequent contamination of the site with virus from a macaque or cells or tissues harvested from this animal. Fomites, contaminated particulates or surfaces, can serve as source of virus as well. In one case, human-to-human transmission was reported and attributed to a shared tube of medication which resulted in contamination at a broken skin site with cream used to treat another patient's bite wound. Later, the same patient autoincoculated one eye during manipulation of a contact lens. In 28 zoonotic cases occurring during the 1980s and 1990s out a total of 46 documented cases confirmed since 1933, 80% have survived infection with the advent of antiviral therapies in contrast to 80% mortality reported in untreated patients. Timely antiviral intervention is an effective means of reducing B virus-associated morbidity and preventing a fatal outcome.

History

The first case to appear in the medical literature was described as follows. A laboratory worker accidentally bitten by a monkey, apparently recovered from the bite, but immediately afterward fell ill of a febrile disease with progressive symptoms of ascending myelitis and died 15 days after the first symptoms of involvement of the central nervous system (CNS). The gross and histological picture included areas of softening in the mid brain and widely diffuse areas of perivascular lymphocytic infiltration. While the brain of this individual was contaminated with a coliform bacterium, it yielded, on rabbit brain passage, a bacteriologically sterile tissue cell culture isolate that killed rabbits with invariably characteristic neurologic symptoms and also caused an exaggerated skin lesion that was subsequently followed by ascending myelitis and death. The virus present in the brains of these animals was thoroughly compared with known strains of herpes viruses by crossed immunity reactions which will be elsewhere described. This agent produced lethargic encephalitis in Cebus but not in rhesus monkeys.

Gay and Holden (1933) reported an ultrafiltrable agent, similar to herpes simplex virus, recovered from this brain tissue as received from Albert Sabin. They initially designated the isolate "W" virus, noting that it caused similar disease in rabbits infected by either intradermal or intracranial routes, but a rhesus macaque exposed to virus showed no illness. Within a year of these first reports by Gay and Holden, Albert Sabin independently reported an ultrafilterable agent that he identified as "B" virus from tissues of this same index patient, naming the virus by using the initial of the last name of the patient. These studies were expanded with the observation that B virus, deadly in this patient, caused disease strikingly different from HSV. Sabin described the case as follows: in 1932, a young physician

(WB) was bitten by a macaque monkey and later developed localized erythema at the site of the animal bite. This apparent localized infection was followed by lymphangitis, lymphadenitis, and ultimately a transverse myelitis, with the demise of WB ascribed to respiratory failure. At the time of WB's death, tissue specimens were obtained for laboratory investigation.

The virus subsequently has been called B virus, herpes B virus, herpesvirus simiae, or Cercopithecine herpesvirus 1. The lethality of B virus infection in rabbits was described by Sabin (1934) who showed that infectivity was independent of route of inoculation. Experimentally infected dogs, mice, and guinea pigs, on the other hand, showed no susceptibility regardless of route of infection. Both Gay and Holden, as well as Sabin, observed that B virus induced immunological reactions in an infected host similar to HSV-1. The virus was noted also to share similarity with pseudorabies as well as with other viruses, including SA8 and two additional herpes non-human primate alpha-herpesviruses recently described, HPV-2, originally described as SA8 (Simian Agent 8)(Eberle *et al.*, 1995) and Langur herpesvirus (J. Hilliard, unpublished data).

Twelve fatalities were identified by 1959 along with five survivors. Detection of antibodies in humans, in the absence of clinical symptoms but with a history of working with macaques, is unreliable; however, these tests early antibody could not discriminate between antibodies induced by HSV types 1 and 2 vs. B virus. Subsequently, with the development of more precise tests, Freifeld *et al.* (1995) observed a high risk population ($n = 325$) that antibodies were rarely, if ever, present in the absence of a history of clinical symptoms. Following another fatal case, however, antibodies were found to be present in at least two coworkers and one additional individual with no previous history of HSV 1 or 2 (J. Hilliard, unpublished data). Two of the three reported a significant illness, describing symptoms commensurate with early stages of B virus infection nearly a decade earlier but with full recovery. These observations suggest B virus may reactivate and be responsible for maintenance of high antibody titers long after acute illness; however, there are too few cases to suggest with certainty that B virus reactivates in humans who survive acute disease.

Distribution in nature

All species of macaques appear to serve as the natural hosts of B virus. There are, in general, genotypic differences associated with B virus depending on the species of macaques from which it was isolated. There is an absence of strong evidence to suggest that there is a difference in the pathogenesic mechanisms of these different genotypes when they infect humans. The macaque host is found most often in the Asian wilds, but colonies of these animals have been exported to a number of other regions, for example, the Isle of Mauritius and Gibralter. B virus can also infect humans (1987, 1987, 1989; Benson *et al.*, 1989; Artenstein *et al.*, 1991), as well as other species of monkeys housed next to macaques (Loomis *et al.*, 1981; Wilson *et al.*, 1990). Other vertebrate species can be infected, including other non-human primates, but in such cases the infected animal serves as a foreign host and frequently succumbs to infection. N/P B virus transmission results from direct contact, whether from animal to animal, animal to person, person to person, or contaminated object to animal or person. There has been only one recognized case of human-to-human transmission observed in 1987 (Palmer, 1987; Weigler, 1992; Weigler *et al.*, 1993).

B virus is highly prevalent in host natural performed reservoirs. Estimations of prevalence have been by a variety of investigators and techniques for both wild and captive macaque populations (Shah and Southwick, 1965; DiGiacomo and Shah, 1972; Kessler and Hilliard, 1990; Weigler, 1992; Freifeld *et al.*, 1995; Sato *et al.*, 1998). Transmission of infection correlates with onset of sexual activity, facilitating transmission of the virus among animals within a group (Weigler *et al.*, 1995). Crowding of animals during transportation seems to accelerate the spread of infection within a community (Keeble *et al.*, 1958; Keeble, 1960). A number of captive colonies worldwide are attempting to define and breed macaques free of B virus, but generally, due to the nature of the virus, antibodies are usually the only measure of prior infection. Investigators will be challenged greatly as they try to eliminate a virus that has coevolved with this host for nearly 30 million years!

As would be expected of an alphaherpesvirus infection in the natural host results is frequently associated with mild clinical signs, if any. B virus infection, however, can have serious consequences under certain immunosuppressive conditions, as observed by a number of investigators and reported by Chellman and colleagues (Chellman *et al.*, 1992). Under these conditions, virus shedding from mucosal membranes can be documented easily by virus isolation in cell culture. In most infected animals, persistent, high antibodies throughout the lifetime of the host provide supportive evidence that B virus, as with HSV, reactivates periodically. N/P Virus can be reactivated in vitro (Boulter, 1975; Vizoso, 1975) from ganglia harvested from asymptomatic seropositve animals. In healthy animals, virus reactivation can be documented by recovery of virus from mucosal samples; however, virus is excreted for only short periods of time (Weigler *et al.*, 1993; J. Hilliard,

unpublished data). Collectively, these observations are not surprising in view of data derived from other alpha herpes viruses.

The virus

Isolation and growth properties of B virus

In 2003, the Department of Justice listed B virus as a Select Agent when it is outside of the natural host further restricting work with this virus, a potential tactical agent of terrorism. Because the agent is capable of causing death in up to 80% of untreated cases, the Center for Disease Control and Prevention (CDC) has undertaken the responsibility of defining agent summary statements for work performed with B virus. Isolation of virus is recommended in BL-3 containment laboratories while growth and propagation is strictly confined to BL-4 maximum containment facilities. Obviously, early work with this agent was done under far less stringent containment.

As noted, B virus was initially isolated from rabbit brain homogenates following fatal zoonotic infections. Within a decade after its discovery, chorioallantoic membranes or embryonated eggs were used for growth and propagation of B virus. By 1954, B virus was reported isolated from primary rhesus kidney tissue used for polio vaccine production. Virus was also noted to be present in rhesus central nervous system tissue. Shortly thereafter, monkey kidney and chick embryo were found to support in vitro replication of virus. In B cells, virus induces syncytial cells uniquely characteristic of this agent.

Following virus isolation, B virus was found to be relatively stable in cell culture media stored at 4 °C. Long-term storage, however, required −80 °C, not −20 °C. These observations have been important for optimizing recoverability of virus from clinical samples to better understand the agent from both the macaque host and others, including humans.

B virus replicates to high titer in cell lines derived from Old World monkeys, particularly in Vero cells derived from African green monkey and vervet kidney cells. Rabbit kidney cells, BSC-1, and LLC-RK also support replication of B virus. Vero cells are an optimal cell line for isolation of virus from clinical specimens. Infected cells balloon, fusing into polykaryocytes that expand outwardly as more cells become infected. Eosinophilic, intranuclear inclusions (Cowdry type A bodies) are observed following fixation and staining of infected monolayers of cells, but inclusions are neither always observed in infected animals nor in some humans with zoonotic infection. As a result, intranuclear inclusion bodies should not be relied upon as a diagnostic marker. In the event this agent is inadvertently isolated in a BL-2 laboratory, cultures should be sealed and forwarded to a registered specialty laboratory that can handle this select agent safely and in accordance with federal guidelines.

In culture, B virus grows with kinetics similar to HSV (Weigler *et al.*, 1993). Virus particles adsorb to cells, resulting in fusion and virus penetration of susceptible cells with suitable receptors. The nature of the cell receptors will be discussed in further detail later. Early in infection (3–4 hours), virus activity eclipses, but cellular responses can be detected by preliminary microarray analysis within the first hour post infection (Zao *et al.*, unpublished data). Although host cell machinery is halted once virus enters the cell, innate immune responses of the cell post infection work presumably to counteract the virus. This, too, will be discussed in more detail later in this chapter. By 4 hours postinfection, DNA synthesis increases dramatically as does synthesis of polypeptides (Hilliard *et al.*, 1987). Morphogenesis of the the virus is also similar to that of HSV, as shown by electron microscopy studies during the time course of infection (Ruebner *et al.*, 1975). Within 6–10 hours after infection, infectious virions are detectable. By 24–28 hours postinfection, intracellular and extracellular virus levels plateau. Similar to HSV, B virus expresses sequential classes of proteins, i.e., immediate early, early, and late proteins (Hilliard *et al.*, 1987). Homologous glycoproteins, as well as structural proteins, are encoded by the B virus genome (Slomka *et al.*, 1995). Previous studies have characterized the antigenic relatedness of many of these proteins to those from HSV types 1 and 2, and to other non human primate alphaherpesviruses (Eberle *et al.*, 1989; Hilliard *et al.*, 1989).

The B virus genome

Two complete B virus genome sequences derived from cynomologus and rhesus monkeys, respectively (Harrington *et al.*, 1992; Perelygina *et al.*, 2003) have been published along with a number of partial sequence analyses (Bennett *et al.*, 1992; Killeen *et al.*, 1992). The total genome length has been calculated to be 156 789 base pairs for rhesus derived B virus and x bp for cynomolgus derived B virus.

B virus contains a double-stranded DNA genome of approximately 162 kbp. One strain of virus originating from a cynomologus monkey has been mapped and subcloned by Harrington *et al.* The genome contains two unique regions (Ul and Us) flanked by a pair of inverted repeats, two of which are at the termini and two internally located, an arrangement allowing four sequence-orientation

isomeric forms. The overall size of the genome is slightly larger than HSV-1 (152 kbp) and HSV-2 (155 kbp). The guanosine:cytosine content of the DNA was calculated to be 75% based on the buoyant density of viral DNA. Eberle *et al.* published data which established the presence in B virus of gene homology to HSV-1 and HSV-2, gB, gC, gD, gE, and gG. Further examination of the sequences of the nine major glycoproteins demonstrated that the 75% of the GC content was conserved within most glycoproteins. Harrington *et al.* showed that the location of genes within the Ul regions of HSV and B virus were collinear; one gene rearrangement was described in an isolate which originated in a cynomolgus macaque. Homologues of HSV Us9 and Us10 genes were noted to be located upstream of the Us glycoprotein gene cluster in contrast to the downstream location of these genes in HSV Us region. This rearrangement was affirmed according to hybridization data and the proposed physical map of Harrington *et al.* Sequence analysis (unpublished) of the prototype strain (E2490) which originated from a rhesus macaque, however, illustrates that B virus DNA is colinear in these same regions with the HSV-1 genomic arrangement (L. Perelygina *et al.*, 2000, unpublished data).

To date, sequences for only a few B virus genes have been submitted to GeneBank, i.e., homologues of gB, gD, gC, gG, gJ, and gI, largely covering the sequence of the Us region. Nonetheless, with a number of laboratories engaged in the sequencing of this virus, the majority of the genome sequence will be accomplished likely within a short time. Each of the glycoproteins for which sequence information is available, except gG, has about 50% identity with HSV, slightly higher for HSV-2 than HSV-1. B virus gG is a homologue of HSV-2 gG and is closer in size to gG-2 (699 kbp) than gG-1 (238 kbp). Glycoprotein sequences demonstrated that all cysteines are conserved as are the majority of glycosylation sites. This conservation suggests that B virus glycoproteins have similar secondary structure to that characterized in HSV. Sequence analyses from these laboratories also suggest that B virus and HSV types 1 and 2 probably evolved from a common ancestor. Using restriction length polymorphisms (RLPs), as a guide, several investigators have shown intrastrain variation among both human and non human primate derived isolates, the significance of which remains to be studied. Eberle *et al.* postulated the possible existence of B virus isolates, which vary with respect to pathogeneicity for non-macaque species, based on the existence of three distinct B virus genotypes found during phylogenetic analyses; however, this postulate must be examined in a suitable animal model. In zoonotic infections, unfortunately little is usually known about the species of most source animals, but where data are actually available many rhesus macaques have been identified targeting this species as the harbinger of a unique, highly pathogenic strain that can cause zoonoses. Published case summaries that implicate other macaque species and in one case a baboon, are difficult, if not impossible to confirm.

Synthesis of viral proteins

Approximately 23 major polypeptides have been identified by electrophoresis in denaturing polyacrylamide gels (Fig. 57.3), but over 50 different polypeptides have been identified by immunoblot analysis. Each has been assigned an infected cell polypeptide number as an initial reference point. The number may be an underestimate of the total produced, but it serves as a basis for comparison in ongoing studies. Molecular weight of these infected-cell polypeptides ranged from about 10 000–250 000 Daltons. Over 75% of the expected coding capacity of the viral DNA was accounted for by these infected cell polypeptides. At least nine bands from electrophoresed infected cell polypeptides containing viral glycoproteins have been thus far identified. Many of these glycoproteins have been cloned and sequenced by two groups. The proteins encoded were mapped to genes by the Us region which was largely colinear to the HSV glycoproteins gD, gI, gJ, and gG, as previously described. Sequence analysis of selected genes show that B virus is most closely related to herpesvirus papio 2 (HVP-2). Although there are protein homologues in herpesviruses of New World monkeys, very little, if any, cross-reactivity exists between B virus and the New World monkey herpesviiruses.

The kinetics of synthesis of the proteins and glycoproteins in infected cells in culture were found to have a course similar to that observed for HSV, although infectious virus was detectable slightly earlier, appearing 6 hours post-infection Both host cell DNA and protein synthesis appeared curtailed during the first 4 hours postinfection. As for glycoproteins, only glucosamine and some mannose were incorporated during the infection in vitro. B virus polypeptides/glycoproteins can be grouped into classes that differ in their relative rates of synthesis at different times throughout the virus replication cycle, as is characteristic of alpha herpesviruses.

Pathology and pathogenesis

During the course of B virus infection, some factors are observed not only in the natural host, but also in the experimentally infected host and the infected human as a result of zoonotic exposure. Those common factors will be

discussed initially, then specific details for each of these host groups will be provided. First, there are various outcomes in infected hosts and evidence can be deduced from the literature that route of infection may play an important role. Specific details are lacking, but some observations can be made. The route of inoculation predicts differences in the time course of infection and spread through the central nervous system and visceral organs, e.g., spleen, adrenal, kidney, and in some cases even heart. Routes of infection are unique to each of the categories of hosts: natural, experimentally infected, and human zoonotic infections. For example, venereal transmission of the virus is common in macaque hosts, whereas intranasal virus can be experimentally delivered to a rabbit, or in the case of zoonotic exposure to a human by accidental aerosolization. However, these routes share one common feature, namely the involvement of mucosal membranes.

The cells that come into contact with the virus initially are another important factor to the permissiveness of the infection. For example, nasal mucosa has been found to be a less ideal site for virus replication than lung. However, nasal mucosa is not entirely resistant since increasing titers of virus can be isolated from these sites. Another important consideration in B virus pathogenesis is the dose of the virus initially introduced. The role of dose remains a challenging issue for study. Both dose and route of inoculation are important factors with respect to the onset of disease. For example, a far greater quantity of virus is required to infect rabbits by aerosolization than by an intradermal route of inoculation, although practically it remains unconfirmed whether these routes and doses parallel human versus nonhuman primate studies. Also, dose may be an important factor in contributing to associated morbidity and mortality. The commonality of each exposure route is generally a mucous membrane. Another common feature of natural, experimental, or zoonotic infection is that B virus can be found in the CNS shortly after the onset of acute infection. But where this virus goes and what it does differs widely in a natural infection versus an infection of a susceptible foreign host, the latter often succumbing to respiratory failure after neurologic deterioration.

The natural host

The macaque, the natural host of B virus, typically suffers little to no morbidity as a consequence of infection. Exceptions appear rarely and seem to involve specific accompanying factors, e.g., immunosuppression. Typically, once a macaque becomes infected following exposure of mucous membranes to virus, infection is relatively self-limiting. The virus may replicate at the site of inoculation and induce a localized erythema. There is also evidence of a limited focal infection of liver and kidney in some macaques. Virus travels via the peripheral nerves subserving the site of inoculation to associated dorsal route ganglia. Latent infection can then be established in the ganglia, with intermittent reactivation of the virus throughout the life of the macaque. In rare cases, viremia has been observed. Virus was also recovered from urine as well as multiple organs. Reactivation from latency has been observed in the natural host as judged by rising antibody titers as well as from recovery of virus by co-cultivation of sensory ganglia with cell monolayers.

During active replication of B virus in the natural host, isolation of virus can be readily accomplished from buccal, conjunctival, or genital mucosa swabs, predictable sites from which an alphaherpesvirus may be recovered during an active infection. The frequency of active infections within a seropositive group of macaques has been observed to be quite low, with relatively brief periods of excretion of virus from mucosal sites. Mucosal ulcers extend down to the papillary layer of the dermis. Two distinct zones have been described, namely a central area of necrosis and a surrounding zone of ballooning degeneration. Around the lesion, "normal" epithelium exists. An eosinophilic polymorphonuclear infiltration characterizes the histopathology of the lesion. Postmortem examination of monkey tissues from animals euthanatized at the time of active virus shedding shows histological evidence of perivascular cuffing of immune infiltrates in sections of spinal cord. Similar examinations of latently infected, healthy animals show no indication of virus from peripheral sites, but virus was recovered from sensory ganglia.

Experimental infections

Rabbits, mice, rats, guinea pigs and chickens have been experimental hosts of B virus as previously mentioned. Disease is not a uniform consequence following inoculation of B virus into mice and guinea pigs; however, several strains of B virus have increased virulence for the mouse. One strain, identified as E2490 was avirulent for white rats and chickens; nonetheless antibody developed after infection. Cotton rats infected by either intraperitoneal, subcutaneous, or intracerebral routes succumbed to infection with selected strains. Rats showed typical hind leg paralysis secondary to transverse meyelitis similar to symptoms in the rabbit. Reagan and colleagues selected, by serial passage human isolates of B virus, strains capable of infecting mice, hamster, and white chicks. With respect to experimental infections, a review of the literature suggests that the rabbit is perhaps the most useful small

animal model since virus replicates in rabbits to high titers, making it a particularly good model for testing antiviral agents.

Using the rabbit, as well as the mouse model, investigators have shown that virus dose was important depending on the route of inoculation. Experimentally infected animals given low doses of intradermal virus developed only erythema that disappeared within a few days and was not associated with further apparent symptoms. In contrast, animals receiving a larger dose developed a necrotic lesion that was generally followed by CNS invasion. B virus subsequently appeared in the regional lymph nodes late after infection. These nodes drained the area of initial infection and with time, necrosis of the infected nodes occurred, as seen upon post mortem. In the CNS focal lesions were seen in pons, medulla, and spinal cord. Spread was most often facilitated by travel through the peripheral nerves, but in rare cases hematogenous route of spread in experimentally as well as inadvertently infected hosts has been described.

Cervical spinal cord and medulla oblongata were the primary sites for virus recovery post mortem. With time post infection, virus was found in olfactory regions of the brain, which may have been due to movement of the virus centripetally through the nerves innervating nasal mucosa. Perivascular cuffing and glial infiltration were characteristic histopathology findings upon examination of brain tissue. Hepatic congestion was accompanied by infiltration of polymorphonuclear and mononuclear cells seen in the periportal areas of the liver. Scattered necrotic foci can be found throughout the lobes of the liver. The presence of inclusions was seen mainly in the regions of inflammation, around pyknotic or karyorrhetic hepatocytes. When lesions were present on skin in the foreign host, the depth of the involved tissue was significantly thicker than that found on mucous membranes, explaining perhaps the reason B virus was recovered weeks or months later from these sites in foreign hosts.

Development of animal models for studies of B virus have been limited by the lack of antiviral drugs or a protective vaccine designed specifically to treat or prevent this infection since there is grave concern for individuals about actively working with this neurovirulent virus should an accident occur even when BL-4 laboratories are available and appropriate protocols are in place.

Human infection

Human infection with B virus generally occurs through an occupational exposure to a macaque shedding virus at a site which comes into contact with broken skin or mucosal membranes. Several cases where no monkey contact occurred in years suggested that virus could be reactivated. Review of all confirmed cases of B virus in humans can be summarized as follows.

The most striking characteristic of human B virus infection is the involvement of the patient's CNS as a target of infection, specifically the upper spinal cord and lower brain.

These areas are the principal sites for virus replication as observed with clinical, laboratory, and post-mortem data, but initially the infected individual generally experiences a flu-like syndrome followed by numbness or parathesias around the site of inoculation. An ascending myelitis occurs during the final stages of the infection in humans, resulting ultimately in respiratory failure. Virus can be recovered at skin sites of inoculation for extended periods of time and viral DNA can be detected generally in cerebrospinal fluid by the time neurological symptoms are experienced. Antibodies can also be detected in the CSF. Generally, death is associated with respiratory complications. Cutaneous lesions, from which B virus can be isolated, sometimes develop late in infection. Edema and degeneration of motor neurons are prominent. Even with advancing disease Cowdry type A eosinophilic intranuclear inclusions can be found in only a few cases, and certainly not uniformly. Gliosis and astrocytosis are late histopathologic findings, thus, there can be evidence of myelitis, encephalomyelitis, or encephalitis, or combinations of each of these conditions.

Pathogenesis of B virus infection has been studied for each of the reported fatal cases and in some surviving cases. With fatal disease, generally, CNS lesions are localized within the upper cervical spinal cord, sometimes extending into the medulla and pons. In some cases hemorrhagic infarcts can be visualized in these areas, whereas in other cases damage appears minimal in spite of the fact that the patient generally succumbs after prolonged ventillatory support. In some cases, patients are alert, but paralyzed and in other cases patients remain in a comatose state which results in respiratory failure. Survivors have varying degrees of morbidity, seen, ranging from little-to-no sequelae to more severe, incapacitating sequelae. Some survivors experience slow progressive neurologic decline, whereas others report few if any effects long term. Several survivors have subsequently given birth to healthy babies with no ill effects for either the mother or infant. Monitoring of the vaginal canal for virus shedding in these individuals prior to delivery has been negative. In several reports, the ocular effects of B virus infection have been reported. Histopathological examination of the patient's eye revealed a multifocal necrotizing retinitis associated with a vitritis, optic

neuritis, and prominent panuveitis. A "herpes-like" virus was identified in the involved retina by electron microscopy in one case. Post mortem vitreous cultures taken from both eyes and retina have been positive for B virus. Thus, B virus can produce infection and destruction of retinal tissue similar to that of other herpesviruses. Ophthalmic zoster-like symptoms have been reported as well and in one particular case, reactivation of latent infection was speculated to have occurred.

To summarize human pathogenesis, the tissues and organs that become infected by B virus vary in some cases perhaps according to the route of infection. If skin is the primary site of infection, the virus usually, but not always replicates in the skin leading in some cases to localized erythema. Knowledge of the site of initial replication is useful for the development of guidelines for disease prevention and also for retrieval of a virus isolate that then allows unequivocal diagnosis. Subsequently, lymphangitis and lymph node involvement are observed. Viremia has not been proven to occur in humans, although with the application of more sensitive assays, e.g., polymerase chain reaction, further insights may be uncovered. With lymphatic involvement, the virus can spread abdominal viscera, where it has been isolated previously. Nevertheless, spread via neuronal routes is the fundamental route of transmission of the virus, as it is with HSV given the involvement of the spinal cord and CNS. Visceral organs, including heart, liver, spleen, lungs, kidneys, and adrenals demonstrate congestion and focal necrosis with variations in the extent of involvement from patient to patient. Recent human cases failed to demonstrate necrosis, but virus was isolated from adrenal, kidney, lung, and liver tissue collected at autopsy. In cases where B virus infection is suspected, medical personnel should follow published guidelines at the time of injury or observation of symptoms of possible infection.

Latency

A characteristic of all herpesviruses is the ability to become latent and reactivate when provoked with the proper stimulus. B virus is no exception. Reactivated infection has been described in both populations of macaques from the wild and captive established colonies. Unequivocal evidence of latent infection caused by B virus in macaques came with studies on frequency of recovery of B virus in monkey kidney cell culture systems. Wood and Shimada obtained six isolates from 650 pools of monkey kidneys, suggesting at least 1% of macaque kidneys contain latent virus that can be reactivated by culturing the cells. Virus was also isolated from rhesus tissues by Boulter and colleagues as well as by cocultivation from a variety of neuronal tissues including gasserian ganglia, trigeminal ganglia, dorsal route ganglia, and spinal cord. Latent virus was also isolated by cocultivation of tissues from experimentally infected rabbits, further supporting the rabbit as a potentially good animal model for B virus infections. Latency likely occurs in human infection as cutaneous recurrences have been documented and there are cases where an individual has not been in contact with macaques for years or even decades but antibodies exist at high levels.

As in HSV infections in humans, a prominent factor associated with reactivation of B virus in the macaque appears to be stress, particularly that associated with the capture and shipment of animals from the wild to captivity. Shedding of virus following reactivation also occurs with illness and during the breeding season of the natural host. No information is yet available on the state of the viral DNA during latency or on the molecular or biochemical events associated with the establishment and reactivation of latent virus.

Epidemiology

Animals

The majority of adult macaques and a very few younger animals in the wild have been reported to be seropositive for antibodies B virus. However, colonies of animals exist in the wild that have been found to be largely seronegative, but each of these colonies was established apart from original natural habitats to meet escalating needs of the scientific community and thus, the epidemiological pattern of virus infection was modified by human intervention. The high seroprevalence in macaques in the wild, the highly infectious nature within captive colonies, and low morbidity in this host confirmed the macaque as the natural host. More recent studies indicate that animals became infected at a higher incidence at the onset of puberty. The increased incidence appears to be associated with sexual transmission within the colony. Infants and juveniles have been reported to demonstrate a very low incidence of infection as judged by the low prevalence of B virus antibodies. Since antibody levels may reflect presence of maternal antibody in animals in contact with dams, it is of importance that both age groups are virus positive, suggesting transmission other than sexual activity occurs.

No particular species of macaque appears to be excluded as a natural host for B virus infection, although there is minimal data available from certain species and absent in

the case of others. Although presence of antibodies has been confirmed in the majority of the different species of macaques, there has been speculation that virus isolated from certain species is less neurovirulent or less neurotropic than virus shed by rhesus macaques. Differences in the restriction endonuclease profiles of the different isolates from different macaque species have been reported, but the lack of available data on the types of macaques involved with each of the documented human cases has not permitted rigorous evaluation of this hypothesis.

Virus shedding during either primary or recurrent infections has been noted to occur at unpredictable frequency with widely variable duration. Analysis of available data indicates that macaques shed virus for a longer duration during primary infection, and for short periods, even hours following reactivation. Levels of shed virus, as measured from mucosal swabs range from 10^2–10^3 pfu/ml in one diagnostic laboratory.

Humans

B virus is an infection that humans rarely contract, but when they do, nearly 80% of untreated cases result in fatality. Epidemiological analysis indicates B virus is usually acquired via zoonotic transmission from either a macaque or infected cells or tissues from the animal. There is, however, one documented case of transmission from human-to-human as previously described, supporting the assumption that B virus can be transmitted similarly to HSV-1 or HSV-2, through mucosal contact with virus which is sometimes present in secretions or wound sites. A recent fatal case which resulted from exposure of ocular membranes to virus from a monkey in the process of being transported refocused attention on an earlier report implicating this type of transmission in the epidemiological analysis of zoonotic transmission of the virus. Current analyses of cases suggest categorization of risk levels with regard to the severity of injury is not useful. The low incidence of B virus infection in humans makes it difficult to reach statistical conclusions, but analysis of cases occuring during the last decade support the fact that first aid of injured or contaminated sites plays a major role in infection control. Only minimal disruption of the protective skin layer or instillation directly to a mucosal membrane can result in initiation of infection when the site is exposed to viable virus. The level of virus needed to initiate an infection in humans remains unknown. Where data are available, rhesus macaques were most frequently implicated as the source of infectious virus in reported human cases, but alone this is insufficient to conclude that the rhesus is uniquely important in the establishment of zoonotic infections. Other species of macaques and even a baboon have been linked to fatal zoonotic infections.

The incidence of zoonotic infections has been correlated retrospectively with periods of increased usage of macaques particularly in biomedical research. Evaluation of past cases underscores that transmission of the virus is often associated with no more than a superficial scratch or puncture, suggesting that once virus gains entry into a host, the ability to initiate disease is perhaps dose independent, at least in some cases. However, this point remains unsubstantiated. Dose and route of entry are topics that require further study to clarify importance in zoonotic infection. Likewise, incidence and prevalence of zoonotic infections must be estimated cautiously since little data regarding the virus source is available from clinically recognized cases.

Host immune responses

Antibodies specific for B virus have been studied in both the natural and the foreign hosts by a wide variety of methods, including serum neutralization with or without complement, competitive RIA, multiple types of ELISA including competition ELISA, and western blot. Limited serial observations have been available from both the natural or foreign host, but comparison of available data have been useful in that relatively consistent responses are induced in both hosts. The time course over which antibodies develop has been measured in wild macaques, captive colony populations, individually imported animals, experimentally infected macaques, zoonotically infected humans, and even in vaccine trial recipients. The ELISA methodologies provide a rapid diagnostic tool with increased sensitivities of detection, and enhanced specificity when competition protocols are utilized. The antibodies which develop in response to herpes B virus infection in both humans and non-human primates are capable of neutralizing HSV-1 and HSV-2, as well as non human primate alpha herpesviruses. The HSV antibodies are not able to neutralize B virus, indicating the presence of virus specific antigens unique to B virus. Sequence data have been useful in confirming the existence of virus specific epitopes.

The humoral immune responses which develop after a B virus infection either in the susceptible host, either natural or foreign, had characteristic patterns of viral antigen recognition throughout the course of infection. The glycoproteins induced antibodies early in the course of infection. Antibodies began to appear within 7–10 days after the infection and consisted of IgM. Within 14–21 days after the onset of acute infection, IgG antibodies were present. In rare cases, the infected host remained persistently

antibody negative in spite of virus isolation. The pattern of the immune response was altered in the cases of humans who have had a previous infection with HSV-1 or HSV-2, since viral antigens that are shared among the three viruses induce an anamnestic response toward shared protein or glycoprotein sequences. Neutralizing antibodies develop in both the natural and foreign host, but at significantly lower levels than the foreign host. The nature and specificity of the humoral responses make it possible to design enhanced serological testing strategies to rapidly identify detectable antibodies and will provide the basis for future diagnostic strategies.

Clinical Manifestations of disease

Observation of the clinical pattern of disease is important for rapid diagnosis of B virus infection in both macaques and humans. In the natural host, recognition of early infection allows removal of infected monkeys from captive colonies that are being established as B virus-free. In B virus-free colonies, it is important to remove seropositive animals and isolate animals with equivocal results to prevent infection of other colony members, or in seropositive colonies to minimize risk to humans who have to handle them. Macaques are not treated with antivirals since the prevalence of infection is generally cost prohibitive. In the case of humans, early recognition of disease facilitates treatment with antivirals, principally nucleoside analogues, a course which appears to significantly lower the morbidity and mortality. Notably, immunosuppression, e.g., administration of corticosteroids, is often associated with reactivated mild disease in the natural host, whereas other agents appear to be capable of facilitating systemic B virus dissemination, culminating in death.

Humans exposed to herpes B virus demonstrate clinically variable signs of infection. Most often, illness after exposure to viable virus is apparent within days to weeks, but in some cases there appears to be a delay in development of acute disease. The reasons for this delay are unknown and, though rare, delays may even range from months to years, making diagnosis difficult. Once symptoms appear, the clinical progression is associated with relatively consistent symptoms, including flu-like illness, lymphadenitis, fever, headache, vomiting, myalgia, cramping, meningeal irritation, stiff neck, limb paresthesias, urinary retention with an ascending paralysis culminating in inability of the patient to maintain respiration, requiring ventillatory support. Cranial nerve signs, e.g., nystagmus and diplopia are also common to most published cases. Sinusitis and conjunctivitis have been observed in some. The array of symptoms may be related to the dose of virus with which the individual was infected or the route of inoculation. Summary descriptions of human cases can be found in two comprehensive reviews.

A summary of reported cases indicates that the highest percentage of deaths post infection occur within weeks postonset. In some cases however life was prolonged artificially for months or years. Incubation times from identifiable exposures to onset of clinical symptoms ranges from days to years, but the majority of cases occur within days to months. Virus has been recovered from throat, buccal, and conjunctival sites, as well as from lesions, vesicles, or injury sites as late as weeks to months post infection. The majority of clinical cases are associated with bites (50%), with fomites (8%) saliva (<5%), and aerosols described as other modes of exposure (10%).

Diagnosis

Non-human primates

B virus infection in macaques is identified by either virus isolation or the presence of specific antibodies or both. The neutralization assay dominated as a diagnostic tool in macaques and humans for many decades. The time required for the results of this test was often a drawback. The dot-blot, RIA, ELISA, and western blots were subsequently developed. Three of these techniques rely on the use of monoclonal antibodies. Each of these tests can be accomplished in less than a day, and are available through commercial laboratories as well as through a national resource laboratory subsidized through NIH's National Center for Research Resources. All of these assays utilize B virus infected cells for antibody detection, making them more effective than other types of assays which rely on HSV-1. This is a particularly important point with respect to diagnostic tools utilized to recognize early laboratory signs of infection for the establishment of B virus-free colonies. Currently, there are no diagnostic serologic tools to identify infected macaques that lack detectable antibodies; however, there are some promising assays being developed for diagnosis of HSV-2 infections in humans that may be adapted for identification of B virus infection in macaques. When selecting an assay for detection of antibodies, the sensitivity and specificity of the test for a specific species of macaque should be known and considered in the final evaluation of the results. Tests dependent on monoclonal antibodies or recombinant reagents should have defined sensitivity and specificity for each macaque species to be tested. Finally, evaluation of a macaque is optimal when

analysis is performed on multiple samples collected at different times, especially in cases when the antibody titer is low (<1:50). A constellation of different tests at numerous time points may be necessary in some cases to correctly determine the status of an animal with low antibody titers, particularly when such an animal is housed in a B virus-free colony.

Virus isolation is the gold standard for diagnosis of infected macaques. Unfortunately, virus isolation is not a particularly sensitive diagnostic tool, with the possibility of many false negatives. Nonetheless, standard cell culture for virus isolation is still a valuable tool for the colony manager and for the veterinarian. Virus positive cultures can be easily recognized with the unique cytopathic effect produced by B virus, but unequivocal confirmation of the identification requires either electrophoretic analysis of infected cell polypeptides or restriction endonuclease digested DNA. More recently, several PCR reactions have been described which can be used to verify the identity of the virus; however, this diagnostic tool is costly for colony management. Nonetheless, when a possible zoonotic infection must be confirmed, PCR may be beneficial for identification of B virus in macaques.

Other species of monkeys become infected infrequently with B virus. These are usually animals that have been co-housed or housed in close proximity to B virus infected macaques at some time. Since many, if not all non human primates harbor indigenous alpha herpesviruses, the important diagnostic point is to differentiate specific antibodies from cross-reactive ones. Euthanasia is generally advised in the case of a B virus infection in a non-macaque monkey since it is likely that the animal will succumb and, in the meantime, would pose a great risk to anyone attempting to treat the infection. B virus has been identified in the patas monkey, colobus monkey, and Debrazza and Thompson *et al.* (in press). In each case, there was a major concern for the people responsible for care of the animal, particularly since these animals often have severe morbidity and are shedding virus. Currently, the most effective assay for diagnosis of B virus in a non macaque monkey would be a competition ELISA to facilitate discrimination between specific and cross-reactive antibodies similar to the challenge faced when diagnosing infection in humans.

Humans

The evaluation of clinical symptoms associated with an antibody or virus positive case is the gold standard for diagnosis of B virus infection in an exposed individual. Both serological and virological techniques are available for diagnosis of B virus infected humans. The CDC has published specified guidelines for recognition and treatment of such infections. In the case of a suspected infection, several emergency resources are available. Contact with the CDC or the laboratories recommended in the CDC guidelines can expedite laboratory support for the clinician suspecting an infection. Generally, a rise in B virus specific antibodies over several days during acute infection can be used to the etiologic agent. However, in other cases, data are equivocal and decisions with regard to the patient must be based on a complex decision table collectively using all diagnostic tools, including clinical symptoms. Virus isolation is again the gold standard for diagnosis, however virus isolation is frequently not possible even under the best of circumstances. Serological diagnosis of B virus in humans is a complex task when an individual with a suspect infection has detectable antibodies as a result of a previous HSV-1 or HSV-2 infection. As discussed in a previous section, significant cross-reactivity exists among these viruses. In the absence of these cross-reactive antibodies, diagnosis is rapid and straightforward, with confirmation using the neutralization assay and/or western blot. This was not the case prior to the development of rapid diagnostic competitive ELISAs and RIAs. The diagnostic tests for humans are performed currently by only a few facilities that have been licensed and have access to BL-4 containment laboratories for the preparation of B virus antigen.

Virus identification can be accomplished by isolation using conventional cell culture, and in clinical emergencies with PCR. The identity of isolates should be confirmed by electrophoretic analysis of infected cell polypeptides or restriction endonuclease digested DNA. The application of PCR is most helpful in the symptomatic patient if virus cannot be recovered. PCR is also a useful tool for monitoring the efficacy of antiviral interventions.

Control of B virus infection

Multiple levels of prevention can be used to prevent B virus infection in both humans and non-human primates, ranging from attempts to eliminate virus to designing methods to work safely in environments where there is increased risk for contracting this agent. The CDC has published detailed guidelines for maximizing protection for individuals working with macaque monkeys. Further, the NIH's National Center for Research Resources has funded the development of B virus-free colonies for NIH-funded research involving these animals in attempt to ultimately eliminate this virus from colonies used for biomedical research. Nonetheless, B virus infected monkeys are plentiful and require

attentive handling adhering to strict guidelines, including barrier precautions.

When B virus is present, it can be inactivated with either heat or formaldehyde. Other inactivators include detergents and bleach. Individuals who work in a decontaminated area should still be alert to injury prevention. Minimizing fomites, however, decreases worker risk and reduces virus spread among animals. One B virus infection in a human was acquired from a cage after sustaining a scratch, underscoring that surface decontamination is important in infection control.

As early as the 1930s, attempts were made to identify an effective vaccine for protection of individuals who could be exposed to this virus while working with macaques or their cells or tissues. Limited vaccine trials were performed in human volunteers and although short-term antibody was induce, it was observed to wane quickly and the vaccine was not pursued further at that time. Recently, a recombinant vaccine was tested and found to induce antibodies in macaques, but the duration of antibodies and protection remain to be studied.

Antiviral therapy has been recognized as an effective prevention of infection progression in humans and animal trials as well, when administered sufficiently early after exposure. Acyclovir and the related family of nucleoside analogues were noted to be effective when given in high doses, e.g., acyclovir at 10–15 mg/kg three times daily for 14–21 days. Efficacy of therapy in cases of infection in humans has been monitored by inhibition of peripheral virus shedding in some cases and by reduction in CSF antibodies or viral DNA load in others. Ganciclovir has a greater efficacy in vitro and thus was used in all proven cases since 1989 with success. Interestingly, prior to 1987, in at least five retrospectively recognized cases, individuals fared well in the absence of antiviral therapy, but overall, the use of acyclovir and ganciclovir remains the recommended therapy by CDC. Generally, antiviral therapy is reserved for human with a clinically apparent infection, however it is also used by an increasing number of facilities for post-injury prophylaxis or after laboratory results indicate an animal may have been actively infected around the time of the exposure. Postinjury prophylaxis has been performed with famciclovir or valcyclovir, as well, both in that have demonstrated efficacy in vitro. Recommendations and guidelines have been published by the CDC, as discussed previously and can be readily accessed. Only a handful of physicians have had experience in the treatment of B virus zoonosis and their participation and expertise were important in the development of the CDC guidelines.

Finally, with respect to prevention, the value of wound cleansing following a potential exposure due to a bite, scratch, splash, or other suspicious injuries is very mandatory. Guidelines for wound cleaning are described in detail in the CDC Guidelines. Every institution working with macaques should have an injury protocol with immediate availability of first aid, a secondary care plan, and last but not least an infectious disease specialist who is a member of the institution's prevention and care response team.

Conclusions

B virus is usually a rapidly advancing, devastating disease which can be interrupted with effective use of antiviral therapies if deployed sufficiently soon after infection. The guidelines for treatment and prevention are widely published and can be rapidly accessed either through CDC or the diagnostic resource using the world wide web address www.gsu.edu/bvirus. Diagnostic techniques are rapidly improving to support clinical diagnosis and information regarding sample collection and evaluation is available at any time to clinical care centers in case of emergencies.

With the newer diagnostic techniques, sensitivity of detection is improving and the barriers posed by the high degree of cross-reactivity among this family of viruses are rapidly being diminished.

Because of the risk of human disease, precautionary methods must be followed in the workplace. Proper attention to the details of housing, management, handling of macaque monkeys, and organized exposure response measures using the CDC guidelines can minimize B virus zoonotic infections. Rapid identification of infection is essential for early inititation of antiviral drug therapy which can prevent further mortality associated with this very interesting alphaherpesvirus.

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Simian varicella virus

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Introduction

After primary infection (chickenpox) in children, varicella zoster virus (VZV) becomes latent in cranial, dorsal root and autonomic ganglia along the entire neuraxis and may reactivate decades later to produce zoster. The incidence of zoster and its attendant neurological complications is related to a natural decline in cell-mediated immunity (CMI) to VZV that occurs with aging, and which also develops in immunocompromised organ transplant recipients, and patients with cancer or AIDS. Yet the mechanism of reactivation and the cascade of events that are precipitated by impaired CMI to VZV are still unknown. To study such events require an animal model of varicella. While experimental animal models of latency and pathogenesis exist for closely related viruses such as herpes simplex types 1 and 2, VZV causes disease exclusively in humans. Thus, lack of a good animal model has hampered the studies of varicella latency and pathogenesis. Several attempts to produce disease by experimental inoculation of animals have led to seroconversion without clinical symptoms (Takahashi *et al.*, 1975; Myers *et al.*, 1980, 1985; Matsunaga *et al.*, 1982; Wroblewska *et al.*, 1982; Walz-Cicconi *et al.*, 1986). Subcutaneous inoculation of the Oka VZV (vaccine strain) into the breast of a chimpanzee has been shown to produce viremia and mild rash restricted to the site of inoculation (Cohen *et al.*, 1996). VZV DNA was detected in blood mononuclear cells (MNCs) of the chimpanzee during the 10-day incubation period. Mild varicella was observed resembling the low-level infection seen in some children vaccinated with VZV, but latency was not studied.

In contrast, simian varicella virus (SVV) causes a natural varicella-like disease of non-human primates. Herein, we describe the biology of SVV, its close similarity to VZV, and its usefulness as a model to study VZV pathogenesis and latency.

Simian varicella virus (SVV) is an alphaherpesvirus that infects Old World monkeys and causes a naturally occurring exanthematous disease similar to human varicella (White *et al.*, 2001). Epidemic outbreaks in African green or vervet (*Cercopithicus aethiops)*, Patas (*Erythocebus patas*), and various species of macaque (*Macaca sp.*) monkeys were first reported in the 1960s and 1970s at five primate centers in both the USA and UK. Virus isolated during these outbreaks was shown to be very similar to VZV in terms of its tissue culture characteristics (Soike *et al.*, 1984a). Clinical (Padovan and Cantrell, 1986; Myers and Connelly, 1992) (Fig. 58.1), immunological (Felsenfeld and Schmidt, 1977,1979) and pathological (Wenner *et al.*, 1977; Padovan and Cantrell, 1986; Dueland *et al.*, 1992) changes produced by SVV infection of primates are similar to those in human varicella. Like VZV, primary SVV infection causes viremia, and infectious virus can be recovered from blood MNCs (Clarkson *et al.*, 1967; Wolf *et al.*, 1974; Soike *et al.*, 1984a). Occasionally, rash becomes hemorrhagic and disseminated (Soike, 1992). Like disseminated varicella in immunosuppressed patients, lung and liver are the most severely affected organs (Roberts *et al.*, 1984). Histological examination of skin and viscera reveals foci of hemorrhagic necrosis, inflammation and eosinophilic intranuclear inclusions (Clarkson *et al.*, 1967; Wolf *et al.*, 1974). Like VZV, SVV becomes latent in ganglia at multiple levels of the neuraxis (Mahalingam *et al.*, 2002).

SVV reactivation has been observed in infected monkeys exposed to social and environmental stress (Soike *et al.*, 1984a). Both the 1968 and 1974 outbreaks of varicella in *Erythrocebus patas* monkeys at the Tulane National Primate Research Center, Covington, LA, were attributed to reactivation of SVV (Soike, 1992). SVV reactivation often appears as a whole-body rash in contrast to VZV reactivation in humans (zoster) which is generally localized to 1–3 dermatomes. Zoster in monkeys is often obscured by fur

Fig. 58.1. Vesicular rash on the abdomen of an African green monkey infected with simian varicella virus. (Reprinted with permission of *Ann. NY Acad. Sci.*)

and the duration of rash is generally less than one week. SVV has been isolated from skin vesicles after reactivation (Fig. 58.2) (Soike *et al.*, 1984a). Identical restriction enzyme profiles have been detected in the genomes of SVV isolated from primary infection and reactivation in the same monkey (Gray and Gusick, 1996). Neither VZV nor SVV has been isolated from blood of otherwise healthy, asymptomatic immunocompetent humans or primates.

Similarities between SVV and VZV

SVV and VZV encode antigenically related polypeptides, and SVV-specific antibodies cross-react with human VZV in serum neutralization and complement fixation tests (Felsenfeld and Schmidt, 1979; Soike *et al.*, 1987; Fletcher and Gray, 1992). Although VZV does not cause disease in non-human primates, it has been used to immunize and protect monkeys from SVV infection (Felsenfeld and Schmidt, 1979). To date, there is no evidence that SVV can infect or cause disease in humans. It seems likely that humans exposed to VZV are protected against SVV infection since nearly all adults in North America are VZV-seropositive and since VZV can protect monkeys from SVV infection.

Simian varicella virus genome

SVV is an enveloped, double-stranded DNA virus. The SVV genome is colinear (Pumphrey and Gray, 1992; White *et al.*, 1997) with that of VZV, sharing similarities in size and structure. The two virus genomes share 70–75% DNA homology (Davison and Scott, 1986; Gray and Oakes, 1984; Clarke *et al*, 1992; Gray *et al.*, 1992). The entire SVV genome has been sequenced, and analysis showed that SVV DNA is 124 784 bp in size, 100 bp shorter than VZV DNA, and its G+C content is 40.4% (Gray *et al.*, 2001, unpublished observations). The left end of the SVV genome contains a small segment (665 bp) that consists of 506 bp of unique sequences flanked on either side by 79 bp inverted repeats; part of this inverted repeat sequence (64 bp) is present at the junction of the long and short segments of the SVV genome. The unique short (U_S) component composed of a 4909-bp region bracketed by 7557-bp inverted repeats (IR_S). The entire short segment of the SVV genome (U_S+IR_S+TR_S) is 147 bp longer than its VZV counterpart. However, the unique short segment (U_S) is slightly shorter than that of VZV.

There are a total of 74 methionine-initiated open reading frames (ORFs) (Fig. 58.3). This includes 71 distinct SVV genes since 3 ORFs (69, 70 and 71) are duplicated within the

repeat regions. The gene organizations of the SVV and VZV genomes are similar if not identical. Of the 70 unique SVV ORFs, 68 share extensive homology with the corresponding VZV genes (Gray *et al.*, 2001). Several SVV genes, including thymidine kinase (Pumphrey and Gray, 1996), uracil DNA glycosylase (Ashburn and Gray, 1999), glycoproteins E (Gray *et al.*, 2001), B (Pumphrey and Gray, 1994), L and H (Ashburn and Gray, 2002) and C (Gray and Byrne, 2003), have been characterized either at the level of RNA or protein and shown to be very similar to their VZV counterparts.

A detailed comparison of the SVV and VZV sequences revealed several important differences at the leftward end (Fig. 58.4): (i) the left end of the SVV genome contains a 660 bp segment that consists of 506 bp of unique sequence flanked on either side by 79 bp inverted segments; (ii) an 879-bp ORF A and a 420 bp ORF (LE) in SVV that are absent in VZV. ORFA has 42% amino acid identity to SVV ORF 4 and 49% to VZV ORF 4; (iii) a 342-bp ORF B in SVV with 35% amino acid identity to a 387-bp ORF located to the left of ORF 1 on the VZV genome; and (iv) a 303-bp ORF in SVV with 27% amino acid identity to VZV ORF 1. No homologue of VZV ORF 2 was detected. Further, all of the known strains of SVV lack a VZV ORF 2 homologue (Mahalingam *et al.*, 2000). Transcripts specific for ORFs A and B have been detected in SVV-infected cells in culture and in acutely infected monkey ganglia. In vitro transcription-translation followed by immunoprecipitation of SVV ORF A showed that it encodes a 35-kD protein (Mahalingam *et al.*, 2000). Thus, the only major difference between SVV and VZV DNA is in the leftward terminus, possibly underlying the species specificity of SVV infection for monkeys.

The U_S of both VZV and SVV DNA invert, resulting in two major isomers of the virus DNA. A small (88-bp) repeat DNA segment brackets the U_L of VZV DNA. Like VZV, ~5% of SVV DNA molecules have an inverted U_L segment in productively infected cells. Further, the SVV genome exists either as circles or concatemers in SVV-infected cells (Clarke *et al.*, 1995), as also shown for VZV (Kinchington *et al.*, 1985). Like VZV, SVV and transfected SVV DNA produce a cytopathic effect in tissue culture, characterized by the formation of syncytia preceding cell lysis, a low virus titer and cell-associated virus (Soike *et al.*, 1984b; Soike, 1992; Clarke *et al.*, 1992).

SVV pathogenesis

Experimental inoculation of SVV in African green monkeys has generated two models of varicella infection. Intratracheal inoculation of 10^4 pfu of SVV into monkeys results in vesicular skin rash at 7–10 days post-infection (p.i.)

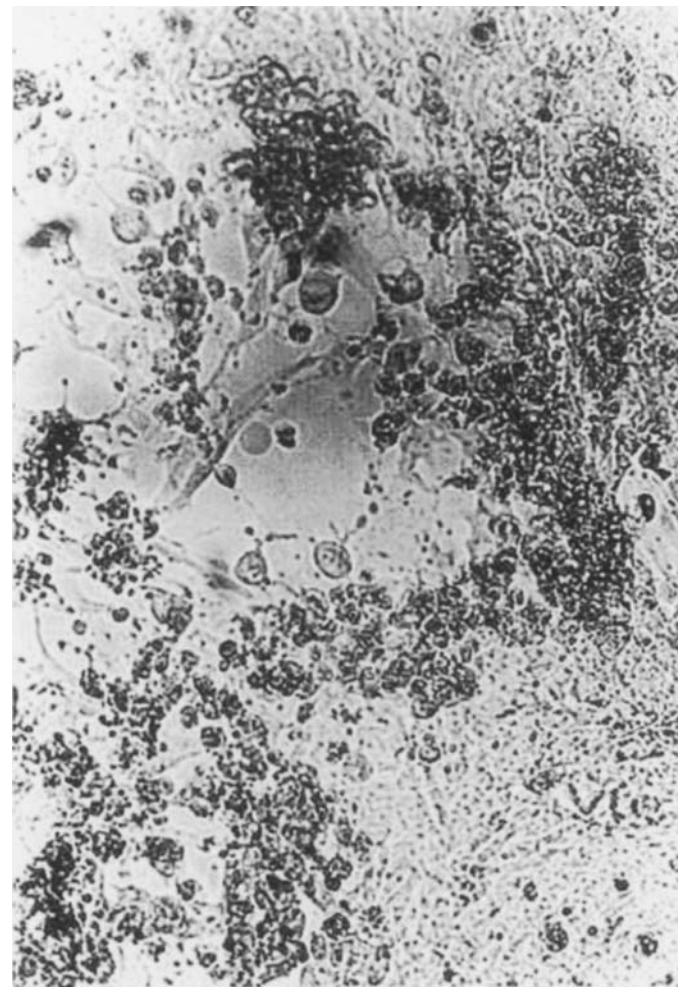

Fig. 58.2. Viral cytopathic effect resulting from infection of Vero (African green monkey kidney) cells using reactivated simian varicella virus isolated from monkeys. (Reprinted with permission of *Ann. NY Acad. Sci.*)

(Figs. 58.1 and 58.5). Viremia can be detected as early as 3 days p.i., peaking at ~5 days p.i., and disappearing by 11 days p.i., indicating hematogenous spread of the virus throughout the body. Hepatitis and pneumonia have been detected at the peak of rash, as suggested in Fig. 58.5. Induction of antibody responses by 12 days p.i correlates with the resolution of rash (Wenner *et al.*, 1977; Iltis *et al.*, 1982; Soike *et al.*, 1984a; Dueland *et al.*, 1992; Gray *et al.*, 1998; Gray, 2003). The time course and route of spread of SVV to ganglia in monkeys after intratracheal or intravenous inoculation was determined by analyzing DNA extracted from monkey tissues 5–60 days later (Mahalingam *et al.*, 2001). SVV DNA was detected in

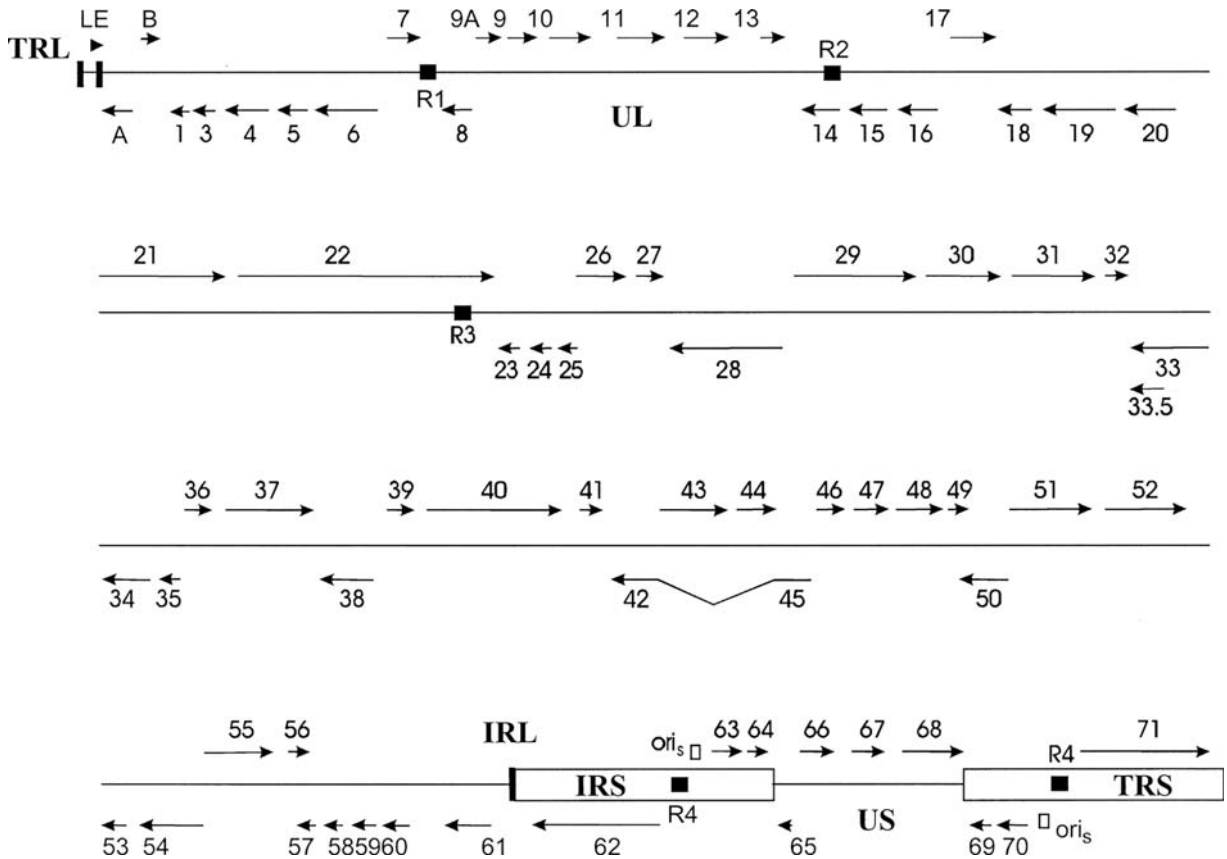

Fig. 58.3. Complete genome map of SVV. The unique long segment (UL) of the genome is flanked by 64-bp of inverted repeats (TRL and IRL). The left end of the SVV genome contains 665 bp segment that contains 506 bp of unique sequence flanked on either side by 79 bp inverted repeats; part of this inverted repeat sequence (64 bp) is present at the junction of the long and short segments (IR_L) of the virus genome. The unique short segment (US) is flanked by 7557-bp of inverted repeats (IRS and TRS). Arrows indicate the direction and location of the 73 SVV ORFS corresponding to the nomenclature conventions of VZV. Black boxes indicate the repeated sequences (R1, R2, R3, and R4). The putative origins of DNA replication (ori_s) are shown. (Reprinted with permission of Virology.)

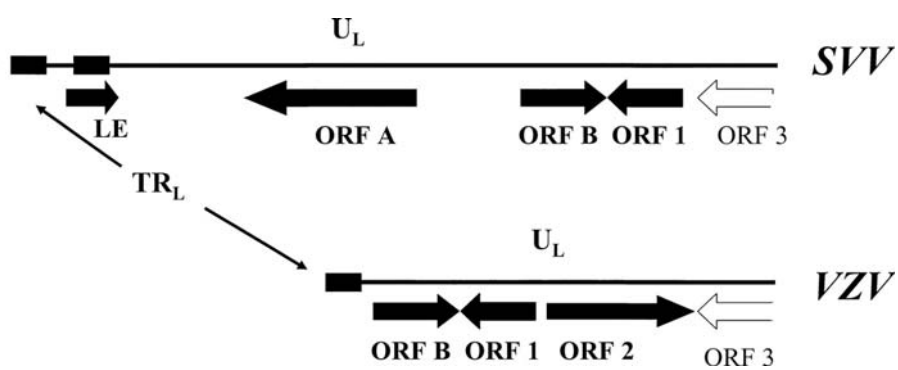

Fig. 58.4. Sequence organization at the leftward end of the SVV and VZV genomes. TR_L indicates the 64- and 80-bp of terminal repeat sequences located at the leftward end of the SVV and VZV genomes, respectively. U_L indicates the leftmost parts of the unique long regions of the virus genomes. SVV ORFs within the 3600-bp SVV EcoRI-I fragment and homologous to the VZV ORFs are shown. ORF B was assigned based on amino acid sequence similarity between SVV and VZV sequences upstream of ORF 1. ORFs LE and A are present in SVV but not in VZV, whereas ORF 2 is present only in VZV. (Reprinted with permission of *Virology.*)

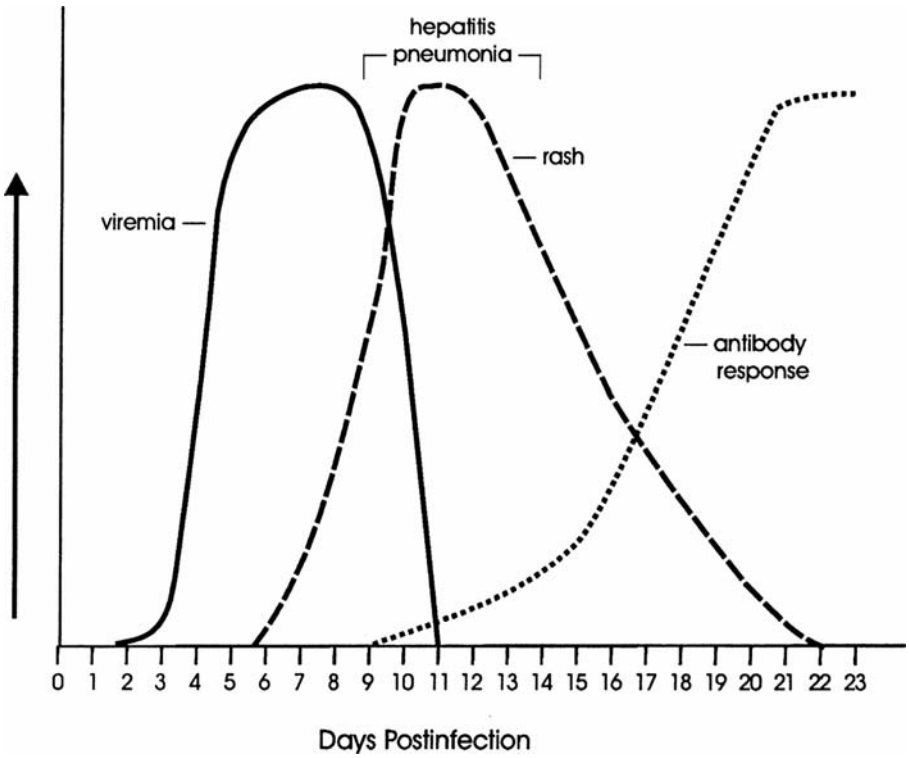

Fig. 58.5. Summary of the clinical course and pathogenesis of experimental SVV infection in monkeys. (Reprinted with permission of *J. Med. Primatol.*)

ganglia of monkeys sacrificed 6–7 days later (before rash). Intravenous inoculation produced more SVV DNA-positive ganglia (63%) than after intratracheal inoculation (13%), pointing to the role of hematogenous spread in ganglionic infection. Like other organs, monkey ganglia become infected with SVV before the appearance of rash (Mahalingam *et al.*, 2001). SVV-specific antigens and nucleic acids have been detected in liver, lung, spleen, adrenal gland, kidney, lymph node, bone marrow, and in ganglia at all levels of the neuraxis (Wenner *et al.*, 1977; Roberts *et al.*, 1984; Padovan and Cantrell, 1986; Dueland *et al.*, 1992; Gray *et al.*, 2002).

Further, experimental intratracheal inoculation of SVV in monkeys results in the persistence of virus DNA for months to years in several tissues, including ganglia, liver and blood MNCs (White *et al.*, 2002a). SVV DNA representing multiple regions of the viral genome has been detected in blood MNCs of SVV-infected monkeys at 7 days and 10 months p.i. (White *et al.*, 2002b), suggesting the presence of most if not all of the virus genome. SVV-infected monkeys that were sacrificed at 10 months p.i., had viremia during acute varicella and SVV may have persisted in blood MNCs. It is also possible that MNCs were being infected while trafficking through tissue where SVV DNA continued to be expressed. Multiple SVV-specific transcripts, including late transcripts, were detected in ganglia from these monkeys (Table 58.1), indicating viral DNA replication and possible assembly of infectious viral particles (White *et al.*, 2002a). Vero cells that were cocultivated with blood MNCs 14 months after intratracheal inoculation did not develop a cytopathic effect, and SVV DNA could not be detected after three subcultivations of Vero cells in tissue culture (White *et al.*, 2002b). It is not clear whether these results represent persistence of the SVV genome as a result of infection of MNCs during acute varicella, or an ongoing abortive infection of MNCs, but the latter appears more likely. In vitro attempts by several laboratories to infect MNCs with VZV have yielded mixed results, but suggest that these cells are only semipermissive for VZV (Arbeit *et al.*, 1982; Gilden *et al.*, 1987; Koropchak *et al.*, 1989; Soong *et al.*, 2000; Zerboni *et al.*, 2000). In all studies, VZV-specific DNA and proteins were detected in T and B cells, monocytes and macrophages, but infectious VZV was recovered primarily from T lymphocytes (Arbeit *et al.*, 1982; Koropchak *et al.*,

Table 58.1. Detection of SVV-specific transcripts in ganglia, lung or liver of virus-infected adult African green monkeys

	Ganglia (months p.i.)				Lung (months p.i.)				Liver (months p.i.)			
ORF	2	5	10	12	2	5	10	12	2	5	10	12
IE 4	3/3[a]	3/3	3/3	1/1	nd[b]	nd	2/2	0/1	nd	nd	0/1	0/1
IE 62	3/3	3/3	2/3	1/1	nd	nd	0/2	nd	nd	nd	0/1	nd
IE 63	3/3	3/3	3/3	1/1	0/3	0/3	2/2	nd	1/2	1/3	1/1	nd
E 21	2/3	3/3	3/3	1/1	nd	nd	2/2	nd	nd	nd	1/1	nd
E 28	2/3	3/3	3/3	1/1	0/3	0/3	0/2	0/1	1/2	1/3	0/1	0/1
E 29	3/3	3/3	3/3	1/1	0/3	0/3	2/2	0/1	1/2	1/3	1/1	0/1
L 40	0/3	3/3	3/3	1/1	0/3	0/3	0/2	0/1	1/2	1/3	0/1	0/1

[a]number of animals positive/number analyzed.
[b]nd = not done.
IE – immediate-early; E – Early; L – late
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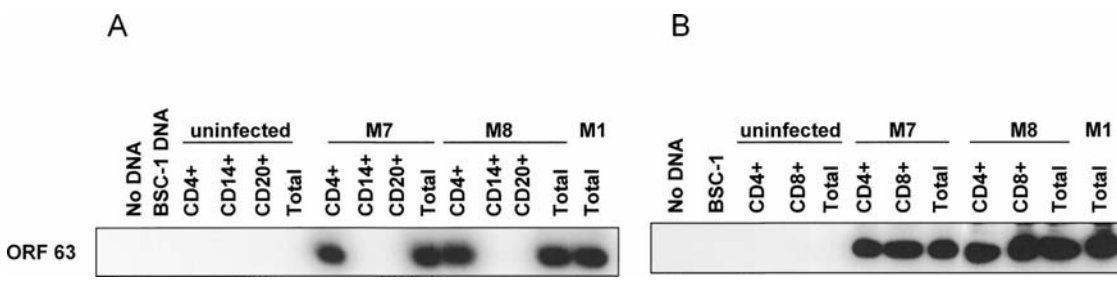

Fig. 58.6. Detection of SVV ORF 63-specific DNA in peripheral blood MNC subpopulations of SVV-infected adult African green monkeys. MNCs from an uninfected monkey and two SVV-infected monkeys (M7 and M8) at 14 months (a) and 23 months (b) after intratracheal inoculation with SVV were sorted by flow cytometry using (a) anti-CD4, anti-CD14 and anti-CD20 monoclonal antibodies and (b) anti-CD4 and anti-CD8 monoclonal antibodies. MNC DNA from the acutely infected monkey (M1) was used as a positive control. DNA extracted from the MNC populations was analyzed by nested PCR followed by Southern blot hybridization. SVV DNA was detected exclusively in $CD4^+$ and $CD8^+$ cells. (Modified and reprinted with permission of *Virology.*)

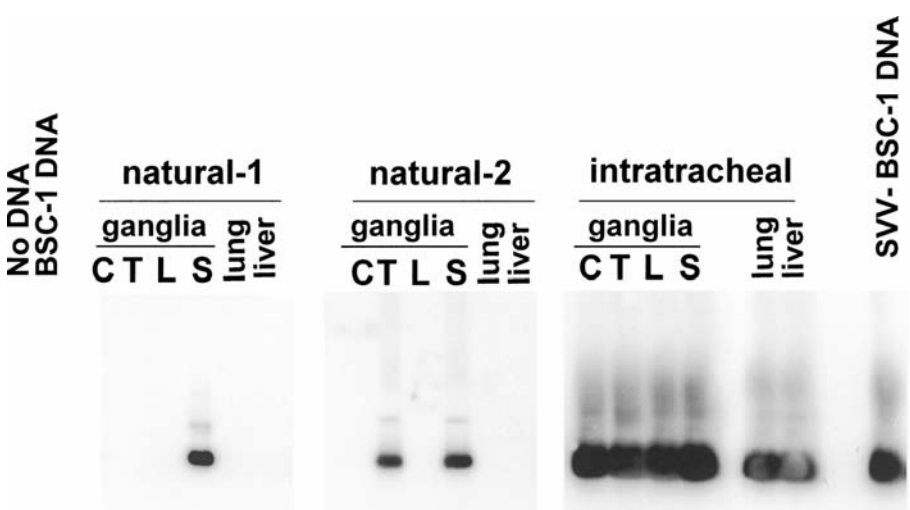

Fig. 58.7. Detection of SVV DNA in ganglia from monkeys naturally infected with SVV. DNA extracted from pooled cervical (C), thoracic (T), lumbar (L), and sacral (S) ganglia, lung and liver from two monkeys (natural-1 and -2) exposed to an intratracheally infected monkey (intratracheal) was analyzed by nested PCR using primers and probes specific for SVV ORF 63. DNA was omitted in one of the reactions (No DNA). DNA from uninfected (BSC-1 DNA) and SVV-infected BSC-1 cells in culture (SVV-BSC-1 DNA) was used as a negative and positive control, respectively. (Modified and reprinted with permission of *J. Virol.*)

1989; Soong *et al.*, 2000). At 14 months p.i., SVV-specific DNA was detected in CD4+ and CD8+ cells, but not in CD14+ or CD20+ cells (Fig. 58.6) (White *et al.*, 2002b). Detection of SVV DNA in blood MNCs likely reflects infection acquired while trafficking through tissue where SVV DNA persists.

The second model of SVV infection in African green monkeys is the simulated natural infection in which SVV-seronegative monkeys are exposed to monkeys previously inoculated intratracheally with SVV (Mahalingam *et al.*, 2002). As noted, monkeys given SVV by intra-tracheal inoculation develop a diffuse rash by 10–12 days after inoculation, and a mild rash also develops at 10–14 days in monkeys caged with the experimentally infected monkeys. SVV DNA is detectable in skin scrapings of the naturally infected monkeys, indicating that the disease is caused by SVV, although SVV DNA is detected only occasionally in the blood MNCs of these monkeys. Six to 8 weeks after the resolution of rash, SVV DNA is detected in multiple ganglia along the neuraxis but not in lung or liver (Fig. 58.7), indicating that latent infection is restricted to ganglia. This monkey model of SVV infection will, for the first time, allow analysis of the extent to which SVV is transcribed during latency and can also be used to dissect, at the molecular level, the cascade of cellular and immune factors in the reactivation process. This is important since VZV reactivation in elderly and immunocompromised individuals produces serious, often chronic and sometimes fatal neurological disease.

Acknowledgments

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Primate betaherpesviruses

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The last few years have witnessed significant expansion of the simian cytomegalovirus (CMV) model of human CMV (HCMV) infection. Progress in the utilization of the simian CMV models has been highlighted by a better understanding of natural history, development of species-specific reagents and techniques, sequencing of several viral genomes, and generation of a bacterial artificial chromosome (BAC) containing a full-length CMV genome. This work has demonstrated that, not only is there strong conservation of genomic organization and coding content, but also that the simian CMV exhibit significant parallels to HCMV in the course of viral infection in both immunocompetent hosts and those without a fully functional immune system. A wide range of experimental approaches into the molecular biology of HCMV, mechanisms of HCMV persistence and pathogenesis, and the design of novel treatment and prevention strategies are now possible in different non-human primate (NHP) models.

Characterization of simian betaherpesviruses has been restricted almost exclusively to CMV. The single report that is consistent with the existence of human herpesvirus (HHV)-6/7-like viruses in non-human primates (NHP) is based on the amplification of a short DNA sequence with nucleic and amino acid homologies to DNA polymerase of HHV-6 and 7 (Lacoste *et al.*, 2000). In contrast, CMV has been isolated from multiple genera and species of old and new world NHP. Each simian species probably harbors its own variant of CMV that has co-evolved with its host during primate evolution. For this review, relevant examples are included from different NHP CMV to describe a monolithic simian CMV phenotype. However, comparative studies between different NHP CMV are extremely limited. There are likely to be important and, as yet, undiscovered variations between the different CMV isolates.

Natural history

Historical evidence of CMV in NHP

The earliest observations of CMV infection in NHP during the first part of the twentieth century were remarkable for their prescient descriptions of CMV–host relationships based entirely on microscopic characterization of the protozoan-like (cytomegalic) cells that had been previously noted in infant humans (Ribbert, 1904; Goodpasture and Talbot, 1921) and guinea pigs (Jackson, 1920). The first published report of NHP CMV occurred three years after Cole and Kuttner discovered that the salivary gland agent of guinea pigs was viral (filterable) in origin (Cole and Kuttner, 1926). In 1929, Stewart and Rhoads (Stewart and Rhoads, 1929) detected CMV cytopathology in tissues from the nasal passages of rhesus macaques (*Macaca mulatta*) acutely infected with poliomyelitis virus (PV). Although there was no PV-associated histopathology, they observed an "intracellular lesion [that] consisted of an acidophilic degeneration of the nuclear chromatin leading to the appearance strongly suggesting inclusion bodies." They also noted that "the lesion is not constant in all monkeys," but did recognize the lesion in the majority of monkeys examined. The lesions were frequently unaccompanied by any signs of a host inflammatory response. Covell extended these findings in 1931 by broadening the tissue distribution of the inclusion-bearing cells and articulating the low pathogenic potential of the probable virus. "I have encountered similar inclusions, not only in the situation mentioned by Stewart and Rhoads but, in addition, in the epithelial cells of the trachea, lungs and bile ducts. [That] such inclusions unaccompanied by symptoms in an animal like the monkey [are] caused by some virus of low virulence is a fair assumption." In 1935, Cowdry and

Scott made the seminal discovery that CMV could establish latency and reactivate from it. They observed that treatment of monkeys with irradiated ergosterol resulted in development of a large number of inclusions in the multiple tissues that were not seen in untreated animals. While it was concluded that ergosterol did not directly cause inclusion formation, the authors suggested that the treatment "may have activated or intensified a process already latent in the kidneys." Since the inclusions had the histological features of those described in humans and other species, they raised the "possibility of a virus being present in the kidney without any attention being called to it by any clinically recognizable symptoms of disease." The authors also concluded that "[the] association between persistence of inclusions and presence of active virus may be stressed."

Taken together, the earliest descriptions of the cytomegalic cells in NHP portrayed the probable virus as a ubiquitous infectious agent with low pathogenic potential that could establish persistent infections, reactivate from a latent state, and remain undetected in apparently healthy hosts. It was not until the first isolation of CMV from an African green monkey (AGM) (*Cercopithecine aethiops*) in 1962 (Black *et al.*, 1963), by one of the groups that had first isolated HCMV in 1956, that reagents became available to explore in detail the natural history and molecular biology of simian CMV.

Seroprevalence

Simian CMV has been described as "adventitious contaminants" during culture of primary simian cells (Smith *et al.*, 1969), a fact that is not surprising given the seroprevalence of CMV in colony-reared animals. Like HCMV, simian CMV is ubiquitous in NHP populations (Black *et al.*, 1963; Swack and Hsiung, 1982; Swack *et al.*, 1971; Andrade *et al.*, 2003; Eizuru *et al.*, 1989; Minamishima *et al.*, 1971; Kessler *et al.*, 1989; Blewett *et al.*, 2001, 2003). In breeding colonies of rhesus macaques, 50% of infants are seropositive by 6 months of age, and almost 100% are seropositive by 1 year of age (Vogel *et al.*, 1994). Similarly high rates of infection have been reported in NHP trapped in the wild, including AGM, rhesus and Japanese (*Macaca fuscata*) macaques, drill monkeys (*Mandrillus leucophaeus*), baboons (*Papio* sp.), and marmosets (*Callithrix jacchus*) (Eizuru *et al.*, 1989; Minamishima *et al.*, 1971; Blewett *et al.*, 2001, 2003; Nigida *et al.*, 1979; Ohtaki *et al.*, 1986; Jones-Engel *et al.*, 2006). The routes of transmission are not known. Virus is probably transmitted horizontally from mother to infant via breast milk and saliva, similar to identified modes in humans (Alford and Britt, 1993). Virus is also excreted in urine, adding another potential route of spread of the virus to naïve cohorts. There are no immunological or virological data indicating vertical transmission, although low rates cannot be excluded (Vogel *et al.*, 1994). Furthermore, there are no reports of spontaneously aborted monkey fetuses or neonates exhibiting histopathologic or clinical sequelae consistent with transplacental CMV infection. Rhesus macaques reach sexual maturity between 2.5 and 3 years, and virtually all breeding-age females are seropositive for rhesus CMV (RhCMV). Based on congenital HCMV infection rates in seropositive humans (Fowler *et al.*, 2003), transplacental transmission of CMV in NHP is likely to be rare ($\leq$1%).

The degree of CMV seroprevalence dramatically changes if animals are reared in smaller cohorts physically separated from seropositive animals. Efforts are underway at the National Primate Research Centers in the United States to develop breeding populations of macaques that are specific pathogen free (SPF) for herpes B virus (*Cercopithecine herpesvirus* 1), an alphaherpesvirus genetically related to herpes simplex virus (HSV) (Huff and Barry, 2003). SPF re-derivation involves separating the infant from the dam at birth, and hand rearing in a nursery. One offshoot of this program has been the recognition that the vast majority of infants are also seronegative for RhCMV and other endemic infectious agents. As long as the seronegative animals are segregated from CMV-infected monkeys, the animals remain CMV-free well past the age of sexual maturity (Minamishima *et al.*, 1971; Nigida *et al.*, 1979).

Multiple strains of CMV are present within each colony for each NHP species. Analyses of independent primary CMV isolates from naturally infected rhesus and Japanese macaques, AGM, and chimpanzee (*Pan troglodytes*) have shown that each isolate possesses distinct restriction fragment profiles (Eizuru *et al.*, 1989; Swinkels *et al.*, 1984; Alcendor *et al.*, 1993). Multiple strains have probably been introduced into each breeding facility by the importation of animals from different locations around the world and the occasional relocation of monkeys between breeding facilities.

Infection in immunocompetent hosts

Pathogenesis

Primary infection of healthy immunocompetent NHP, either by natural routes of exposure or experimental inoculation, does not result in overt clinical signs of disease. Since infection can naturally occur within the first months of life, simian CMV has low pathogenic potential, even in infants. Transient hematological changes, such as lymphocytosis, monocytosis, and, neutrophilia, are observed in

some rhesus macaques following intravenous inoculation, but not orally inoculated monkeys (Lockridge *et al.*, 1999). Mononucleosis is an uncommon but clinically important outcome of primary HCMV infection (Alford and Britt, 1993) that has not been associated with CMV infection in non-human primates. Recurrent infection is similarly unremarkable in terms of clinical outcomes. As noted above, the earliest postmortem descriptions of CMV in monkeys revealed that a large percentage of healthy animals had cytomegalic cells in the absence of overt disease (Stewart and Rhoads, 1929). The presence of the inclusion-bearing cells is now the exception. In recent years, inclusions have been rarely observed in tissues of immunocompetent monkeys. The reasons for this apparent change in frequency are not known, but may be related to improvements in colony management practices that may have reduced stresses in the animals. There is only a single description of recurrent CMV disease in NHP not associated with immunodeficiency or immunosuppression. Eight of 12 chimps euthanized in 1955 for a variety of clinical conditions were found to have characteristic CMV inclusions in the parotid and submaxillary glands (Vogel and Pinkerton, 1955). Three of the eight were further found to have CMV inclusions associated with focal to extensive areas of inflammation and necrosis in the adrenal cortex. One chimp also had a prominent myocarditis together with numerous cytomegalic cells. Although there was no evidence of immune suppression in these chimps, many were infected with *Mycobacterium tuberculosis* and enteric bacteria, which may have sapped their immune vigor, resulting in activation of CCMV.

Viral dynamics of primary infection

Since seroconversion begins long before the age of sexual maturity, primary infection most likely occurs via the oral mucosa following ingestion of virus-positive breast milk or saliva. The general course of primary infection involves rapid bloodstream dissemination from the site of infection to multiple tissues throughout the body. In monkeys naturally exposed to RhCMV, viral DNA can be PCR amplified from plasma coincident with the earliest detection of antiviral antibodies (these authors, unpublished). Although the time of exposure to virus can never be precisely defined in natural infections, the kinetics of CMV DNA in blood in relation to host immune responses suggest that spread of the virus through the blood is a normal component of early infection. This pattern of detection of viral DNA in plasma is recapitulated in experimental infections. Animals inoculated either intravenously (IV) or orally with RhCMV usually develop peak copy numbers of viral genomes in plasma at 7 days post inoculation, coincident with the development of anti-RhCMV IgM (Lockridge *et al.*, 1999). Monkeys inoculated IV with 10^5 to 10^6 plaque forming units (PFU) of RhCMV characteristically reach approximately 10^4 and 10^6 genome equivalents per milliliter of plasma within 5 and 7 days, respectively (Sequar *et al.*, 2002; Chang and Barry, 2003). Plasma CMV DNA levels decline after 7 days, although the rate of decline is variable between monkeys. Some become CMV DNA negative by 3 weeks post-inoculation, while others exhibit a more gradual decline with low copy numbers (1000/ml of plasma) present out to as long as 11 weeks. After its initial clearance, viral DNA is rarely detected in plasma. The presence of viral DNA or virus in the blood is not always observed with other forms of inoculation. Intraperitoneal injection of AGMCMV into rhesus macaques did not result in viremia, although the animals remained viuric for over two years (Swack and Hsiung, 1982). Similarly, subcutaneous inoculation of RhCMV efficiently results in systemic spread of virus, but RhCMV DNA is frequently undetectable in plasma (these authors, unpublished).

By the time CMV DNA is first amplified from plasma, the virus has probably disseminated throughout the body. RhCMV DNA can be detected in oral and genital swabs within two weeks of IV inoculation (these authors, unpublished) and in multiple tissues by 2 weeks (Lockridge *et al.*, 1999). Viral DNA is usually detectable in the spleen and frequently from axillary and inguinal lymph nodes, kidney, bone marrow, and liver (Lockridge *et al.*, 1999; Sequar *et al.*, 2002). In addition, the parotid and submandibular glands appear to be preferred sites after natural exposure to virus (unpublished). In acutely infected animals, lymphofollicular hyperplasia and neutrophilic splenitis are the most prominent histopathological changes observed in IV and orally inoculated animals (Lockridge *et al.*, 1999). Although no cytomegalic cells have been observed following experimental inoculation, cells expressing the viral immediate-early 1 (IE1) protein have been observed in the spleen of IV-inoculated monkeys (Lockridge *et al.*, 1999; Chang and Barry, 2003). The vast majority of antigen-positive cells following acute infection are localized to the perifollicular regions, with lower numbers of IE1-positive cells observed within the germinal centers or in the red pulp. The location of IE1-positive cells within the spleen changes over time such that, by 6 months, the majority of staining cells is within the red pulp.

Immunological parameters of primary infection

The development of viral-specific immune responses is rapid and increases in intensity as viral plasma DNA loads

decrease (Lockridge *et al.*, 1999). Antibodies to IgM become detectable 1 to 2 weeks post-inoculation (p.i.) and usually become undetectable by 4 to 8 weeks. Anti-CMV IgG develop 2 to 5 weeks p.i. and continue to increase for a period of 3 to 6 months. Neutralizing antibody titers follow the same kinetics as CMV-specific responses. Affinity maturation of antiviral IgG, measured as an increase in avidity, continues for a period of at least 6 months, even if antibody titers reached a plateau 3 months earlier. Antiviral antibodies are directed against multiple viral structural and non-structural antigens, although each monkey develops a distinct pattern of reactivity on western blots. Antibodies develop to glycoprotein B (gB) (Yue *et al.*, 2003) and the RhCMV equivalents of phosphoproteins (pp) 28, 65, and 150 (Yue *et al.*, 2006), as well as to other structural and non-structural proteins soon after infection (Vogel *et al.*, 1994). The region of RhCMV gB corresponding to the AD-1 region of HCMV gB is highly conserved and especially immunogenic (Kropff and Mach, 1997; Kravitz *et al.*, 1997), and can be neutralized by monoclonal antibodies specific to the AD-1 region of HCMV gB (Kropff and Mach, 1997). Analysis of gB-specific and neutralizing titers in rhesus macaques indicates that gB encodes a large proportion of neutralizing epitopes, but not all (Yue *et al.*, 2003). The NHP CMV encode all of the counterparts of the HCMV envelope glycoproteins (Davison *et al.*, 2003; Hansen *et al.*, 2003; Rivailler *et al.*, 2006), and some of these, such as gH and the gM/gN complex, may represent additional targets of neutralizing antibodies, similar to HCMV (Mach *et al.*, 2000; Urban *et al.*, 1996).

Cellular immune responses to total RhCMV antigens and to the IE 1 and 2, and pp65 proteins of RhCMV are detected as early as 2 weeks following experimental CMV infection in rhesus macaques and generally precede the onset of antibody responses (A. Kaur, unpublished data). The magnitude of the CMV-specific cellular immune responses increases with time and by 6 months to 1 year have reached levels comparable to those seen in naturally infected macaques. Cytolytic and cytokine-secreting $CD8^+$ T-lymphocytes specific to RhCMV IE1, IE2, and pp65 and the CMV-encoded interleukin-10 (cmvIL-10) are readily detected in long-term CMV-infected rhesus macaques (Kaur *et al.*, 2003; Pitcher *et al.*, 2002). A. Kaur, unpublished data). In addition, $CD8^+$ T lymphocyte-mediated cytolytic activity to an unidentified early protein presented by rhesus CMV-infected fibroblasts is detected in CMV-seropositive macaques (Kaur *et al.*, 1996). Although robust $CD4^+$ T lymphocyte responses to total RhCMV antigens are detected in the majority of CMV-seropositive macaques (Kaur *et al.*, 2002), the immunodominant target specificity of this response has not yet been determined.

Virological and immunological parameters of chronic infection

Two salient features characterize the persistent phase of infection: chronic viral shedding and stability of the antiviral immune responses. It has long been recognized that healthy, infected monkeys can remain viuric for many years following primary infection (Swack and Hsiung, 1982; Asher *et al.*, 1974). In a cross-sectional survey of three subspecies of colony-reared and wild-caught baboons, baboon CMV (BaCMV) was isolated from approximately 50% of the animals (Blewett *et al.*, 2001), a detection rate that is similar to colony-reared rhesus macaques (these authors, unpublished). For BaCMV, virus was isolated almost exclusively from saliva and throat swabs and only rarely from genital swabs or urine. Molecular techniques for the detection of RhCMV DNA have yielded similar results. In a longitudinal study involving repeated sampling, some monkeys had amplifiable RhCMV DNA in oral and/or genital fluids at multiple timepoints over a 30-day period (Huff *et al.*, 2003). A cross-sectional analysis of adult males indicated that approximately 50% were DNA-positive at any one time. In contrast to the clearance of CMV DNA from the blood within a few weeks, the presence of infectious virus and/or viral DNA in mucosal fluids demonstrates that there is active and ongoing virus replication at mucosal surfaces, probably for the life of the infected host.

Active viral gene expression at mucosal surfaces does not necessarily engender phenotypic changes or inflammatory responses at the site of infection. Antigen-positive cells can be detected occasionally by immunohistochemistry in tissues such as the spleen (Lockridge *et al.*, 1999) and salivary glands (these authors, unpublished data). What is especially noteworthy is that the tissues are frequently histologically normal, without any cellular infiltrate in response to viral antigen production. The level of viral replication is sufficient, presumably, to enable horizontal transmission to naïve cohorts, while at the same time minimizing tissue destruction and host immune responses. At the level of the infected host, therefore, it can be stated that CMV is a persistent virus because of the chronic, ongoing production of viral antigens and progeny virions. At the cellular level, however, CMV can clearly establish, maintain, and recrudesce from a quiescent state. Based on the high frequency of animals that shed virus and the social dynamics of NHP populations, seropositive animals should be regularly challenged with heterotypic strains of virus that may be immunologically distinct from the variant associated with primary infection. It is unknown whether prior immune responses restrict subsequent challenge following natural exposure. Seropositive monkeys can be reinfected

following experimental RhCMV inoculation with as little as 100 plaque forming units of a subsequent prolonged viuria of the challenge virus (L. Picker and J. Nelson, unpublished data).

The relatively constant exposure to CMV antigens (endogenous and exogenous) probably explains the pattern of antiviral immune responses observed in long-term infected monkeys. End-point IgG titers to total viral antigen preparations hover around the plateau level achieved at the end of the primary infection (Yue *et al.*, 2003; Baroncelli *et al.*, 1997). Although there can be a wide range of antiviral/neutralizing antibody titers and avidity indices observed amongst infected animals, only minor fluctuations occur over time within any one monkey. However, different results can be observed if antibody responses to individual viral antigens are assayed longitudinally. While antibody responses to gB usually parallel those to total RhCMV antigens, a minority of monkeys exhibit changes in gB reactivity while total antibody responses remain unchanged (Yue *et al.*, 2003). Cellular responses to RhCMV antigens also appear to be relatively stable over time. In one study, the frequency of $CD4^+$ T-cells secreting IFN-γ following stimulation with RhCMV antigens remained within a twofold range over a 6-month period (Kaur *et al.*, 2002). Similar to antibody titers, a wide range of CMV-specific $CD4^+$ responses occurs within a cohort of infected monkeys.

The stability of the virus-host relationship, exemplified by the absence of disease, places a relatively high immunological burden upon the infected host. One study of healthy, CMV-positive rhesus macaques observed that 0.16%–5.8% of total $CD4^+$ T-lymphocytes were CMV-specific (Kaur *et al.*, 2002). In a cross-sectional analysis of eight healthy CMV-seropositive macaques, 0.4% to 8% of peripheral $CD8^+$ T-lymphocytes were specific for CMV antigens (A. Kaur, unpublished data). Comparably high frequencies of CMV-specific memory T-cells have been observed in persistently HCMV-infected humans (Kern *et al.*, 2002; Gillespie *et al.*, 2000; Sylwester *et al.*, 2005). In contrast to humans, however, IE1 but not pp65, appears to be the dominant protein recognized by CMV-specific $CD8^+$ T-lymphocytes in rhesus macaques (A. Kaur, unpublished data).

Infection in non-immunocompetent hosts

Retroviral-induced immunodeficiency

The first published descriptions of fulminant CMV disease in NHP were in the context of rhesus macaques naturally coinfected with the immunosuppressive simian type D retrovirus (SRV). Beginning in the late 1960s and occurring through the early 1980s, periodic outbreaks of an acquired immunodeficiency disease were observed in macaque species housed at the California and New England Primate Centers (Henrickson *et al.*, 1984; London *et al.*, 1983; Henrickson *et al.*, 1983; Letvin *et al.*, 1983a,b; Osborn *et al.*, 1984; King *et al.*, 1983). The disease was characterized by persistent lymphadenopathy, severe wasting, chronic diarrhea, high morbidity and mortality, and multiple opportunistic infections, including activated CMV. Because of the strong immunological and pathological similarities between immunodeficient macaques and the emerging human AIDS, intense efforts were initiated to isolate the etiological agent of the so-called simian AIDS (SAIDS). The great majority of the SAIDS cases were due to the spread of SRV from healthy carriers that were unknowingly infected with this immunodeficiency-inducing virus. Subsequently, simian immunodeficiency virus (SIV) was isolated at the New England Primate Center from a few immunodeficient rhesus macaques that were free of SRV (Gardner *et al.*, 1994).

The presence of cytomegalic cells containing cytoplasmic and/or intranuclear inclusions in tissues from immunodeficient monkeys, often associated with tissue necrosis and neutrophilic infiltration, bore almost all of the hallmarks of CMV disease in human AIDS patients. The incidence of CMV disease in monkeys with SAIDS caused by SIV or SRV can be variable, but upwards of one-third to one-half of CMV seropositive animals exhibit evidence of CMV activation at necropsy (Kaur *et al.*, 2003; Osborn *et al.*, 1984; King *et al.*, 1983; Kuhn *et al.*, 1999; Baskin *et al.*, 1988). As with HCMV, simian CMV can produce end-organ disease in different tissues, including central and peripheral nervous system, lung, lymph nodes, liver, gastrointestinal tract, and arteries (Baskin, 1987). Depending on the thoroughness of sampling, CMV pathology can be detected within multiple tissues of an animal or may be limited to just a single site. To date, CMV retinitis has not been reported in a SAIDS monkey. A single published report describes the electron micrographic detection of herpesvirus-like particles within the eyes of two SIV-infected, RhCMV seropositive macaques (Conway *et al.*, 1990). The reasons for the absence of detectable CMV retinitis in monkeys are not known. They may be related to the relatively rapid onset of SAIDS (within 1–2 years of SIV or SRV inoculation) and the frequent early termination of experiments to spare the animals excess pain and suffering.

The activation of RhCMV infection during SAIDS pathogenesis is similar to that of HCMV during human AIDS and stands in marked contrast to the status of the virus

during persistent infection in immunocompetent hosts. In addition to the increased frequency of cytomegalic cells, other changes include an increased frequency of detectable viral DNA in blood, elevated genome copy numbers in tissues, and declining measures of anti-CMV immune functions, such as CTL activity, cytokine secretion, and neutralizing antibody titers (Sequar *et al.*, 2002; Kaur *et al.*, 2003). The magnitude and timing of the changes can be used to predict those animals most at-risk for developing RhCMV disease. The kinetics of these perturbations are variable, dependent, in part, on the relative timing of CMV and SIV infection.

If SIV infection occurs during the primary phase of RhCMV infection, the onset of RhCMV disease and SAIDS is both accelerated and more severe, compared to older monkeys that are persistently infected with RhCMV at the time of SIV infection (Sequar *et al.*, 2002). Five juvenile rhesus macaques inoculated with SIV two to four weeks after RhCMV infection (either experimental or natural exposure) died with SAIDS or with early lymphoid depletion within 10–25 weeks of SIV inoculation. Three of the monkeys required euthanasia between 10 to 15 weeks post-SIV and died with histological evidence of RhCMV disease in multiple tissues. Compared to controls infected with only RhCMV, all five coinfected animals had elevated RhCMV genome copy numbers in plasma and tissues at the time of necropsy. A different pattern of RhCMV infection emerges when the time interval between RhCMV and SIV infection is increased. When monkeys persistently infected with RhCMV are inoculated with SIV, the range in the time of death post-SIV is greatly extended (10 – >72 weeks) (Sequar *et al.*, 2002; Kaur *et al.*, 2003). One study observed that SIV-positive monkeys with histologically confirmed RhCMV disease have significantly shorter median time of death (17 weeks) than monkeys without RhCMV disease (57 weeks) (Kaur *et al.*, 2003). The same study also noted significant associations between the potential for RhCMV disease and changes in virological and immunological markers of RhCMV infection. Monkeys that had multi-organ disease due to reactivated RhCMV had large increases in RhCMV plasma DNAemia and lower neutralizing antibody titers at death, compared to the monkeys without histological evidence of RhCMV disease. In addition, there were significant reductions over time in RhCMV-specific $CD4^+$ and $CD8^+$ T lymphocytes in those animals with RhCMV sequelae. The declines in immune competence in these monkeys appear to have been specific to RhCMV. No significant correlations were observed between changes in anti-RhCMV antibodies and those specific to the rhesus rhadinovirus and lymphocryptovirus (related to the human herpesviruses, human herpesvirus 8 and Epstein–Barr virus -EBV, respectively) (Kaur *et al.*, 2003).

A second study has also observed that specific changes in parameters of RhCMV infection can be used to predict the occurrence of RhCMV disease in SIV-infected monkeys. Four of five animals with RhCMV sequelae exhibited at least two of the following characteristics prior to death: (a) either a failure to develop an increase in anti-RhCMV antibody avidity or a decline in avidity over time, (b) a progressive decline in anti-RhCMV antibody titers, and/or (c) prolonged detection of RhCMV DNA in plasma (Sequar *et al.*, 2002). Immunodeficient monkeys without RhCMV disease had no more than one of these characteristics.

Many papers published since the advent of HIV and AIDS have speculated on the role of HCMV in augmenting HIV pathogenesis (Drew *et al.*, 1985; Robain *et al.*, 2001; Peterman *et al.*, 1985; Webster, 1991). Studies of RhCMV and SIV coinfection are consistent with the notion that activated RhCMV can enhance progression to SAIDS (Sequar *et al.*, 2002; Kaur *et al.*, 2003). Rigorous experiments demonstrating a causal effect of RhCMV on SIV pathogenesis are still required. One study has noted a possible direct effect of CMV on HIV-1 infection in chimpanzees (Castro *et al.*, 1992). Two chimps seropositive for chimpanzee CMV (CCMV) were infected with HIV-1. PBMC cultures had been persistently negative for isolation of HIV over a 3–4-year period. After intrarectal and/or intravenous inoculation with CCMV-infected human fibroblasts, HIV was recovered from the PBMC from both chimps. One was positive for HIV isolation six times over a 12-month period, and HIV was recovered once from PBMC from the other chimp. Two chimps similarly treated with uninfected fibroblasts did not reactivate HIV.

Transplantation

The onset of CMV sequelae in NHP is not limited to immunodeficiency but can also be a factor in another clinically relevant parallel of HCMV pathobiology. CMV disease has been observed in immunosuppressed NHP receiving either allografts or xenografts, although the number of published descriptions is limited to date. The frequency of activated CMV appears to be related to the intensity of the immunosuppression regime. Xenograft recipients, such as either rhesus- or pig-to-baboon, that undergo treatments designed to prevent acute rejection (e.g., anti-thymocytic globulin – ATG) can develop CMV histopathology similar to HCMV disease in human allograft recipients, including pneumonitis and vasculopathies (Teranishi *et al.*, 2003; Mueller *et al.*, 2002; Ghanekar *et al.*,

2002). Prophylactic antiviral therapies common in human transplant recipients are not routinely employed in NHP studies. In the case of the pig-to-baboon xenograft, viral histopathology occurred only in the species-appropriate tissues; no cross-species disease was observed (Mueller *et al.*, 2002). The mechanisms of CMV reactivation in these studies are poorly defined, primarily because the studies were not designed initially to identify causal relationships to transplant-associated CMV disease. A potential association with recipient CMV serostatus was noted in one rhesus-to-rhesus renal allograft study (Pearson *et al.*, 2002). When monkeys were treated with antibodies to block the CD40/CD40 ligand signaling pathway, disseminated CMV disease was seen only in the CMV seronegative recipients ($n = 3$, seropositive donor), but not in 15 seropositive recipients.

Two early studies provide intriguing hints of potential stimuli for triggering reactivation of CMV. In the first study, CMV seropositive cynomolgus macaques were treated with a regimen of three immunosuppressive agents (ATG, cyclophosphamide, and cortisone acetate) given multiple times over 18 days (Ohtaki *et al.*, 1986). With this treatment protocol, 2 of 6 monkeys exhibited only a limited number of cytomegalic cells following necropsy 21 days after initiation of immunosuppression. If the animals were infected with varicella zoster virus (VZV) 3 days after the first immunosuppressive injection, 100% of the monkeys ($n = 11$) developed severe and systemic reactivation of CMV, similar in pathology to HCMV disease in human transplant recipients. CMV inclusions were observed in multiple tissues, and 50% of the animals developed pneumonia. Other tissues demonstrated evidence of CMV vasculitis, consistent with a critical role of the endothelium in simian CMV reactivation. None of the animals exhibited evidence of VZV disease. Similar rates of CMV reactivation were observed when immunosuppressed monkeys were injected with formalin-inactivated VZV, demonstrating that active VZV replication was not essential for the development of CMV lesions (Ohtaki *et al.*, 1988).

The utility of NHP as experimental models to study transplantation-associated CMV disease remains to be determined, based on the fact that the frequency of CMV disease in allograft and xenograft recipients is unknown. There are multiple examples in the literature of long-term studies of simian xenografts (>300 days post-transplant) occurring in the absence of clinical or histopathological evidence of reactivated CMV. Based on the transplant literature, it appears that the incidence of CMV disease in NHP is lower than in human allograft recipients. Whether this is due to sampling error or differences in reactivation potential following immunosuppression is unknown.

Immunosuppression associated with measles virus

CMV activation and disease can also be observed following viral, non-iatrogenic immunosuppression. Macaques are highly susceptible to infection by measles virus, a paramyxovirus that can induce temporary immunosuppression in non-SIV/SRV-infected monkeys (McChesney *et al.*, 1997; Willy *et al.*, 1999). Measles virus pathogenesis in NHP is similar to infection in humans, ranging from subclinical, to mild (skin rash), to fatal infection. Disseminated CMV infection (lung, lymph node, and stomach) has been detected in Japanese macaques that died of measles virus infection (Choi *et al.*, 1999).

Fetal Infection

RhCMV can cause a range of developmental and growth defects in rhesus macaque fetuses similar to those observed in human infants congenitally infected with HCMV. However, the published studies of CMV-induced fetal disease in rhesus macaques required direct inoculation of fetuses with virus in utero. Until transmission across the placenta can be demonstrated (either natural or experimental), fetal infection in NHP should be viewed as a model of intrauterine pathogenesis. Taking advantage of timed matings in NHP, it is possible to establish the time of conception to within two days (Tarantal, 1990; Tarantal and Hendrickx, 1988a,b). Using ultrasound guidance, needles can be directed through the abdominal wall of the dam to deliver virus to precise locations within the developing fetus at defined stages of gestation. Growth and developmental outcomes can be prospectively monitored by ultrasound, and fetal samples (blood, amniotic fluid, and tissue) can be obtained by needle biopsy (Tarantal, 1990; Tarantal and Hendrickx, 1988a,b).

Inoculation of rhesus macaque fetuses with RhCMV has been done via the intra-amniotic (IA), intracranial (IC), and IP routes from late in the first trimester through mid-gestation (Tarantal *et al.*, 1998; London *et al.*, 1986; Chang *et al.*, 2002). Severe developmental anomalies were observed in approximately 50% of inoculated fetuses, independent of the route of inoculation. Some fetuses, however, are developmentally normal even with inoculating titers as high as 10^6 PFU delivered in the late first trimester (Barry *et al.*, 2006). The developing CNS appears to be especially sensitive to CMV disease, with a spectrum of developmental abnormalities ranging from focal lesions to severe bilateral anomalies (Tarantal *et al.*, 1998; London *et al.*, 1986; Chang *et al.*, 2002). These include microcephaly, lissencephaly, ventricular dilatation, leptomeningitis, encephalitis, and periventricular calcifications, all

hallmarks of congenital HCMV infections. Some of the neuropathological changes, especially lissencephaly and microcephaly, are consistent with early insults to CNS development (Barkovich and Lindan, 1994; Hayward *et al.*, 1991; Twickler *et al.*, 1993). The periventricular zones and the choroid plexus are early targets for RhCMV infection following IC inoculation of RhCMV (Chang *et al.*, 2002). The systemic distribution of RhCMV-infected cells soon after IC inoculation supports the hypothesis that RhCMV readily crosses the blood–cerebrospinal fluid barrier to sites of susceptible neuronal stem cells and protoneurons. The cortical malformations observed in the inoculated fetuses may have resulted from an early cytopathic effect that initiated a cascade of defects in proliferation, migration, and organization.

Although RhCMV histopathology was limited to just the brain in one study (London *et al.*, 1986), other sequelae have usually been observed, including intrauterine growth retardation and systemic RhCMV disease (Tarantal *et al.*, 1998; Chang *et al.*, 2002; Barry *et al.*, 2006). Placental abnormalities (deciduitis, infarction, calcification, and lymphocytic infiltration) have been seen in some inoculated fetuses (London *et al.*, 1986).

Molecular biology

As more simian CMV sequences have become available on GenBank, it is now possible to extend the comparisons of their coding content and genetic organization with that of HCMV to include simian–simian analyses. In general, the strong parallels between the natural histories of the primate CMV are reciprocated at the structural and genomic levels, although important interspecies differences exist. An overarching issue in comparing CMV genomes is the consideration of what factors have driven adaptation of a CMV species to its particular host. Since viruses are obligate intracellular pathogens, CMV evolution is driven by virus–host interactions. Commonalities amongst and distinctions between primate CMV can be viewed as a reflection of the similarities and variations of selective pressures between the different but closely related primate hosts.

For any genetic element to be fixed within the population of any CMV species, it must have conferred a selective advantage during evolution from a progenitor CMV. This is equally applicable to both coding and non-coding portions of the genome. Regions of cross-species sequence identity, therefore, represent those domains where significant sequence divergence would produce a selective disadvantage. Conversely, highly divergent domains can be considered as focal points for evolutionary adaptation necessary to maintain optimal replication of the virus during host speciation. What might distinguish the macro- and microselective pressures between hosts is essentially unknown. Comparisons of CMV natural histories indicate that genetic changes have not come at the expense of altering the unifying concept of the CMV-host relationship. That is, CMV is a virus with low pathogenic potential that establishes a lifelong persistence in an immunocompetent host.

Virion structure

The basis for inclusion in the *Herpesviridae* family is the structural conservation of a linear double-stranded DNA genome packaged within an icosadeltahedral capsid (100 to 110 nm in diameter) composed of 162 capsomers. The nucleocapsid is surrounded in turn by a featureless tegument and a viral glycoprotein-studded envelope (Roizman and Pellet, 2001). By definition, the simian CMV adhere to this rule, and they exhibit little structural distinction from HCMV (Fig. 59.1). Mature virus particles and distinct capsid forms are observed by electron micrograph in infected cells. The variant capsid structures include the A (empty capsid, no DNA), B (scaffolding proteins but no DNA), and C (mature capsids containing DNA) forms characteristic of HCMV and HSV (Blewett *et al.*, 2001, 2003; Lee *et al.*, 1988). The RhCMV virion is identical in size to that of HCMV (220–230 nm), whereas those of BaCMV and drill CMV are slightly smaller (140–230 nm) (Blewett *et al.*, 2001, 2003). The range in sizes of these latter two simian CMV species is apparently due to variability in the size of the envelope. Similarly, the B-capsid form of AGMCMV is almost identical in size (inner radius = 49.5 nm) with that of HCMV (50 nm). Density mapping of the AGMCMV B-capsid demonstrates that the architecture (i.e., T = 16 triangulation geometry, capsomer shape, and intercapsomer triplexes) is strongly conserved with other herpesviruses (Trus *et al.*, 1999; Butcher *et al.*, 1998; Chen *et al.*, 1999). Some distinctions in structure have been observed between the cytoplasmic forms of the B capsid of AGMCMV and HCMV, although the differences may have resulted from the loss of some protein constituents during purification (Trus *et al.*, 1999; Chen *et al.*, 1999).

Genome coding content

The genomes of the RhCMV (Hansen *et al.*, 2003; Rivailler *et al.*, 2006) and CCMV (Davison *et al.*, 2003) genomes have been fully sequenced and described in the literature, as have selected regions of the AGMCMV genome. A partially completed sequence of BaCMV has been deposited on GenBank (AC090446), although there has been no published analysis of it, yet. There has been strong conservation of coding content, nucleotide and amino acid sequences,

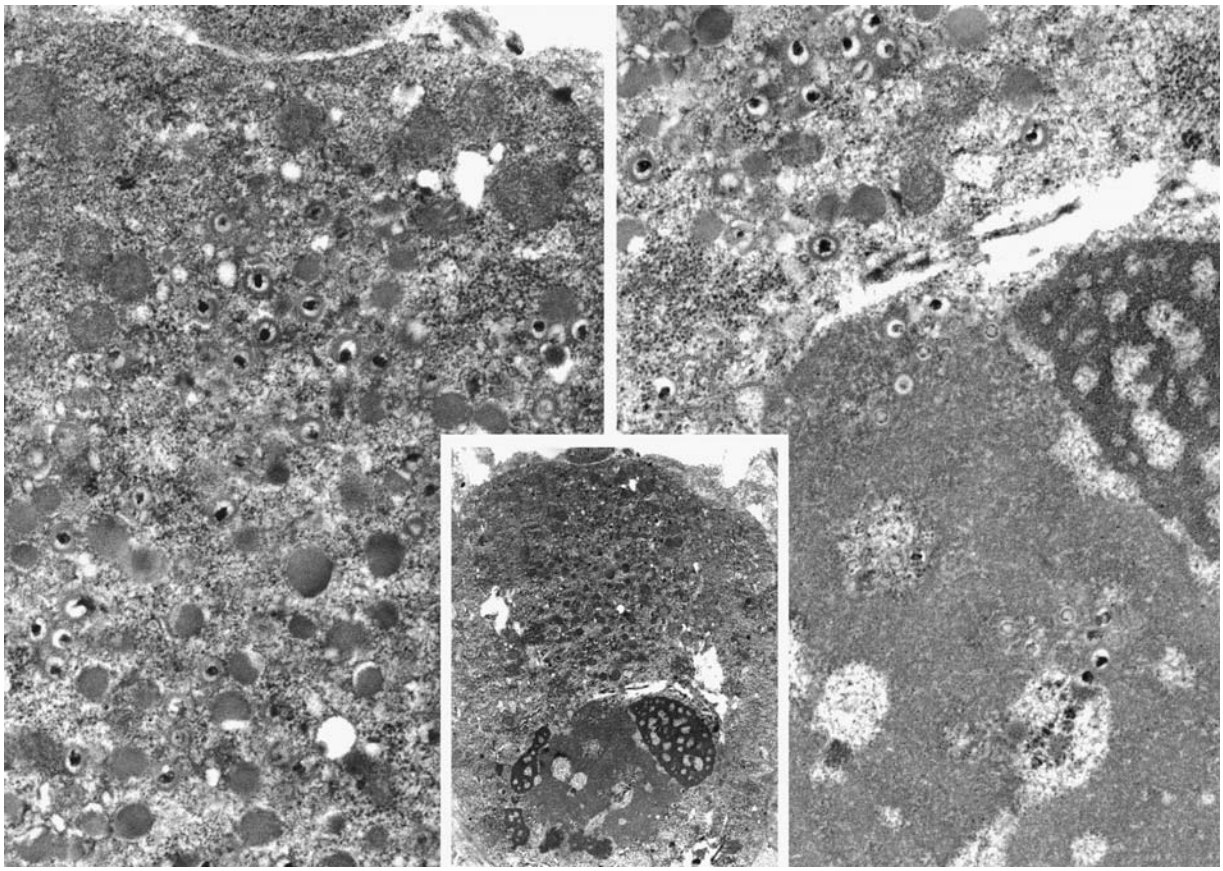

Fig. 59.1. Electron micrographs of RhCMV virions in a cochlear cell of a rhesus macaque fetus. A rhesus macaque fetus was inoculated intracranially with RhCMV strain 68–1, and the cochlea was removed and processed for electron microscopy 25 days later (S. Tinling, A. Tarantal, P. Barry, unpublished). A low magnification (center) and two high magnification (right and left) images of a cytomegalic cell of unknown type are presented illustrating nuclear and cytoplasmic forms of the RhCMV capsid and virion, respectively.

and linear genetic order amongst the primate CMV following the divergence and speciation of their hosts. Noted distinctions are observed, however, between the primate CMV. These include the selective addition (or loss) of genes within one CMV genome relative to the others, and the high rate of interspecific (but not necessarily intraspecific) sequence variation of some viral proteins that bind to highly conserved cellular proteins.

The coding content of HCMV is still open to debate 13 years after the original analysis of the AD169 sequence predicted 208 ORF (Chee *et al.*, 1990). One recent study compared potential ORF of HCMV and CCMV reasoning that legitimate coding regions would likely have been conserved between viruses isolated from the two most closely related primate hosts. This led to the recognition of 145 ORF in the tissue culture adapted AD169 strain of HCMV, 164–167 in wild-type HCMV, and 165 in a CCMV strain that was isolated from an adult chimp (Davison *et al.*, 2003). A subsequent study, using an algorithm that evaluates coding potential by looking for pattern relationships of HCMV amino acid sequences to the Swiss-Prot/TrEMBL database of proteins (Murphy *et al.*, 2003a), predicted that AD169 encodes 192 proteins. Finally, comparisons of clinical isolates of HCMV with each other and with tissue culture adapted strains have led to the conclusion that there are 252 ORF in clinical isolates, almost 30 of which have not been described before (Murphy *et al.*, 2003b).

The vast majority of ORF in HCMV have counterparts in the CCMV and RhCMV genomes. Two RhCMV genomes have been sequenced and annotated. One sequence (GenBank accession number AY186194) was derived from purified virion DNA of a low passage strain (68-1, available from ATCC) that had been cultured on primary rhesus fibroblasts (Hansen *et al.*, 2003). The other sequence (GenBank

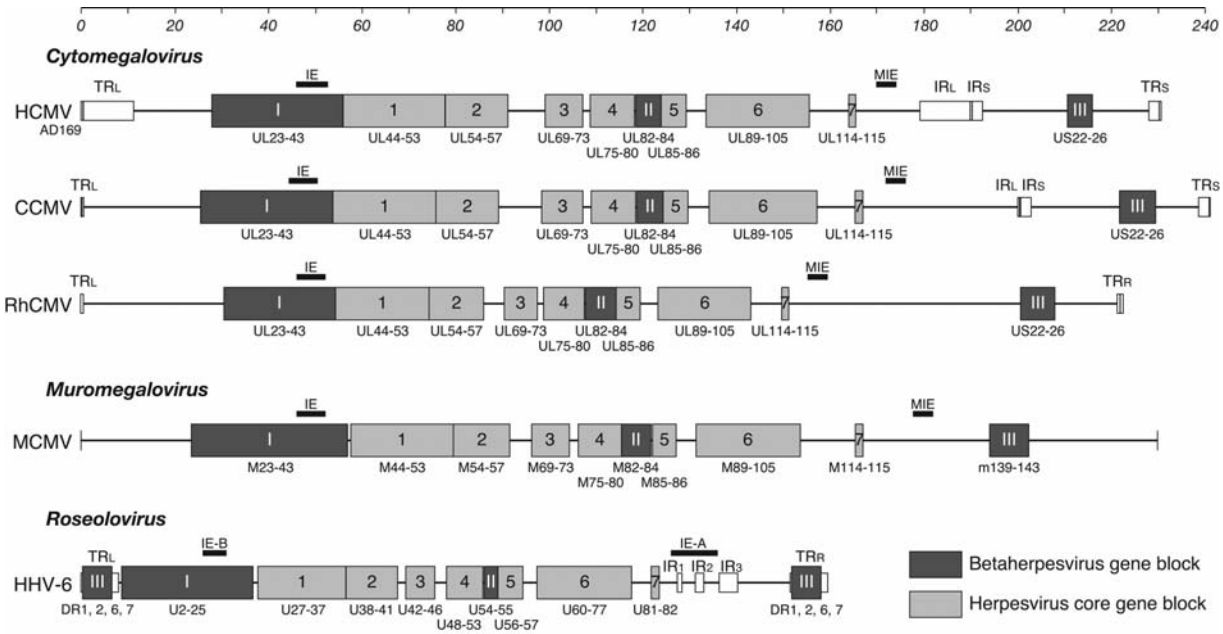

Fig. 59.2. Schematic illustration of the genome structure and gene arrangement of representative viruses comprising the Subfamily *Betaherpesvirinae* and two non-human primate CMV. The size scale at the top is shown in kbp. The horizontal lines represent unique regions of the viral genomes. Open rectangles represent reiterated sequences in the viral genomes. TRL, IRL, IRS, and TRS denote terminal or internal inverted repeats flanking the UL and US components, respectively. For RhCMV and HHV-6, TRL and TRR denote terminal direct repeats at the left and right termini of the viral genomes, respectively. Filled rectangles represent sequence blocks consisting of genes in similar orientation and/or encoding homologous amino acid sequences. The sequence blocks of conserved core genes among the herpesvirus family (block 1 though 7, shown as light grey rectangles) and conserved genes found only in betaherpesvirus subfamily (block I through III, shown as dark grey rectangles) are diagrammed. Corresponding ORF within each conserved gene block are listed. The locations of two complex IE loci within each viral genome are marked with black bars. Gene nomenclature and location are from the following references: HCMV AD169 (Davison *et al.*, 2003; Chee *et al.*, 1990), CCMV (Davison *et al.*, 2003), MCMV (Rawlinson *et al.*, 1996), and HHV-6 (Gompels *et al.*, 1995). The nomenclature for RhCMV genes (Hansen *et al.*, 2003) is based on the layout shown in Fig. 59.3(b).

accession number DQ120516) corresponds to a RhCMV (strain 180.92) isolated from an SIV-infected macaque that had been passaged six times in human fibroblasts and seven times on rhesus fibroblasts (Rivailler *et al.*, 2006).

The 68-1 strain, first isolated in 1968 (Asher *et al.*, 1974), contains a 221 459 bp genome that potentially encodes 230 ORF of 100 or more contiguous amino acids, each of which possesses a translation initiation codon (Hansen *et al.*, 2003). Some of the RhCMV ORF have no apparent equivalent in the HCMV and CCMV genomes, and it is not known whether they represent expressed genes. The total of 230 RhCMV genes should be considered provisional at this point in time and subject to reinterpretation with subsequent analysis. The 180.92 strain genome (215,678 bp) potentially encodes 258 ORF and includes 8 additional ORF not found in 68-1 and 34 ORF that were not listed in the characterization of 68-1 (Rivailler *et al.*, 2006). The 180.92 strain lacks 10 ORF that are present within the 68-1 genome. Expression analysis has not been performed to determine whether the potential ORF are, in fact, expressed. Whatever the final tally, it is evident that evolution of RhCMV in its rhesus host has resulted in coding capacity not found in either HCMV or CCMV (described below).

Importantly, the sequences of both the 68-1 and 180.92 genomes are consistent with the interpretation that there have been deletions and rearrangements during in vitro passage. Comparison of the 180.92 sequence with that of 68-1 (Rivailler *et al.*, 2006) led to the recognition that there is a discontinuity between the genomes within the portion of the genome corresponding to the ULb′ region of HCMV. ULb′ represents a labile region of the HCMV genome that is deleted and rearranged during tissue culture adaptation, such that the intact sequence is found in clinical isolates of HCMV and not in strains that have undergone extensive passage in vitro. The fact that there is the same lability within the corresponding region of the RhCMV genome

indicates that deletion and rearrangement can occur within a limited number of serial passages in tissue culture.

Genome structures

A hallmark of herpesvirus genomes is the conservation of its sequence organization. Based on the copy number, location, and orientation of repeat elements, herpesviral genomes can be grouped into six classes, designed A to F (Roizman and Pellet, 2001). Three types of genome structure have been identified in the members of *Betaherpesvirinae* (Fig. 59.2). HCMV and CCMV possess a complex type E genome, consisting of two unique components (UL and US), each flanked by inverted repeats RL and RS, respectively (Davison *et al.*, 2003). The size of CCMV repeat elements flanking the UL component (TRL and IRL) is considerably smaller than the AD169 and Towne strains of HCMV, but is similar to those of Toledo and low-passage clinical isolates (Prichard *et al.*, 2001). The RhCMV, AGMCMV, and BaCMV genomes each consist of one unique sequence with no internal repeat elements subdividing the genomes into two components (type F genome) (Chang and Barry, 2003; Hansen *et al.*, 2003; Hayward *et al.*, 1984; Rivailler *et al.*, 2006) (E. Blewett, unpublished data). The RhCMV genome contains a 750-bp sequence element from the left end of the genome variably repeated ($0 - \geq 4$) at the right end of the genome (predominantly one or two copies) (Chang and Barry, 2003), similar to the termini of the guinea pig CMV (GPCMV) genome (McVoy *et al.*, 1997). The RhCMV genome does not undergo genome inversion at the junction between the UL and US genes (Chang and Barry, 2003).

Gene arrangements

Based on the completely sequenced betaherpesvirus genomes (Davison *et al.*, 2003; Hansen *et al.*, 2003; Chee *et al.*, 1990; Nicholas, 1996; Cha *et al.*, 1996; Gompels *et al.*, 1995; Rawlinson *et al.*, 1996; Vink *et al.*, 2000), there are several fundamental principles of betaherpesvirus genetic architecture (Fig. 59.2). These relate to the extent of genetic conservation amongst the herpesvirus family members. Primate CMV genes can be grouped into those present (a) in all herpesviruses, (b) only in betaherpesviruses, and (c) only in primate CMV. Genes in CCMV have been named based on their sequence and positional homology with HCMV (Fig. 59.3(a)) (Davison *et al.*, 2003). Both murine and rat CMV (MCMV and RCMV, respectively) exhibit a simple genome structure (type F) with ORF colinear only to the UL region of HCMV genome (Fig. 59.2). The nomenclature for MCMV and RCMV ORF numbers them from the left to the right end of the genome (Rawlinson *et al.*, 1996; Vink *et al.*, 2000). The numbers of the homologous ORF are arranged to be congruent with the HCMV numbering system for the UL region. ORF with sequence homology to HCMV ORF are indicated by uppercase prefixes (M or R), whereas those not conserved in HCMV genome are designated with lowercase prefixes. As described by Hansen *et al.* (2003), RhCMV genes are sequentially numbered beginning at the first ORF at the left end of the genome. To emphasize the higher level of genomic colinearity between members of Genus *Cytomegalovirus* for this review, RhCMV ORF are designated according to their HCMV homologues (Fig. 59.2 and 59.3(b)). The original designations (Hansen *et al.*, 2003) are also presented in Fig. 59.3(b).

The core genes inherited from the herpesvirus family progenitor are located within seven core-gene blocks in the central region of the viral genome (Fig. 59.2) (Roizman and Pellet, 2001). Core genes include those encoding proteins involved in nucleic acid metabolism, DNA replication, and virion structure and maturation (Table 59.1). Sequence analysis of the 68-1 strain of RhCMV did not identify a full-length UL71 gene due to the presence of an apparent single base insertion (Hansen *et al.*, 2003). However, a highly conserved UL71 ORF is present within BaCMV (AC090446; E. Blewett, unpublished data) and the 180.92 RhCMV isolate (Rivailler *et al.*, 2006), although both predicted peptides are shorter than the UL71 of either HCMV or CCMV. The UL22-33 block of genes was originally considered to be betaherpesvirus-specific (Roizman and Pellet, 2001). Analysis of the HCMV and CCMV genomes has led to the recognition that the UL22 ORF does not represent a true gene (Davison *et al.*, 2003). With more betaherpesvirus sequences now available, betaherpesvirus-specific gene blocks (beta-blocks) can now be expanded to include UL23-43 (block I), UL82-84 (block II), and US22-26 (block III) (Fig. 59.2). These gene clusters are retained in the viral genomes across all three genera of *Betaherpesvirinae* with identical order and polarity along with the core-gene blocks, except that beta-block III is within the direct repeat regions of HHV6/7 and at the left end of the tupaia herpesvirus genome (Bahr and Darai, 2001). Included within these are three unique gene families (UL25, UL82, and US22) and one G-protein coupled receptor (UL33) that have no counterparts in the genomes of alpha- or gammaherpesviruses. In addition to the seven pan-herpesviral core gene blocks and three beta-blocks, two complex IE loci are positionally preserved within the betaherpesviral genomes with high similarity in both structures and splicing patterns (Davison *et al.*, 2003; Nicholas, 1996; Rawlinson *et al.*, 1996; Colberg-Poley *et al.*, 1992; Chang *et al.*, 1995; Barry *et al.*, 1996; Nicholas, 1994; McCormick *et al.*, 2003; Schiewe *et al.*, 1994) (Fig. 59.2).

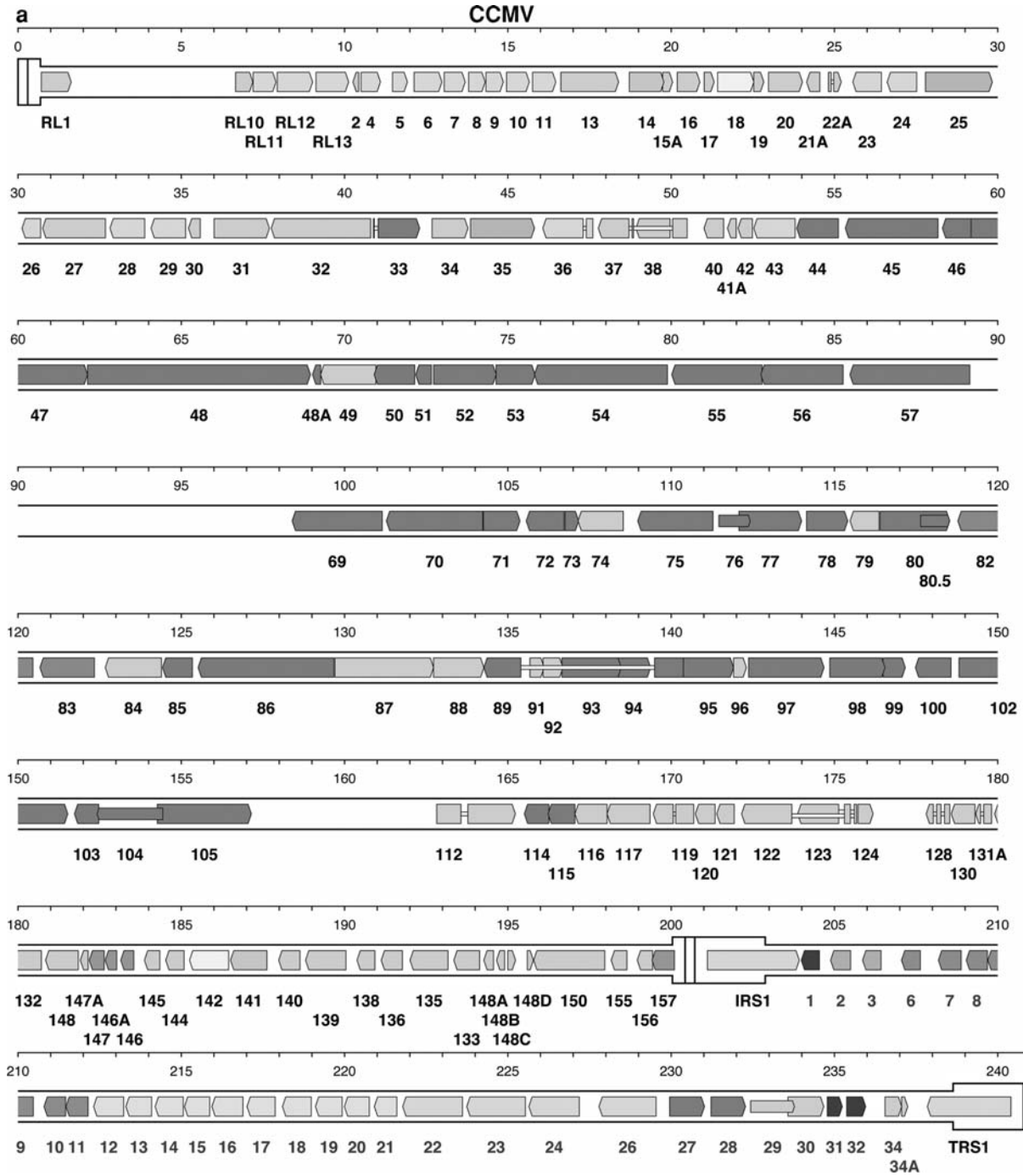

Fig. 59.3. Layout of the genes in the (a) CCMV and (b) RhCMV genomes. The size scales are in kbp. Reiterated sequences (TRL, IRL, IRS, and TRS of CCMV; TRL and TRR of RhCMV) are shown in a thicker format than the unique components of the viral genomes. Colored arrows with gene nomenclature listed below denote the order, orientation, and gene family (grouped according to the key) of predicted protein-coding regions within each viral genome. Narrow white bars represent introns connecting the coding exons. The prefixes of genes corresponding to those in HCMV UL region (shown in black text) and US (shown in blue text) have been omitted. Genes corresponding to those in HCMV AD169 RL and RS regions are given their full nomenclature. Colors differentiate between conserved core genes in the Family *Herpesviridae* with subsets of non-core genes, grouped into unique gene families, conserved in Subfamily *Betaherpesvirinae*, or Genus *Cytomegalovirus*. The nomenclature system for HCMV and CCMV genes is applied to the homologous genes within the RhCMV genome. The RhCMV ORF originally described in Hansen *et al.*, 2003 are listed within each colored arrow. RhCMV ORF with no apparent HCMV homologue are not listed in this figure and can be found in the original manuscript. (a) modified with permission from reference (Davison *et al.*, 2003) (copyright 2003, The Society for General Microbiology).

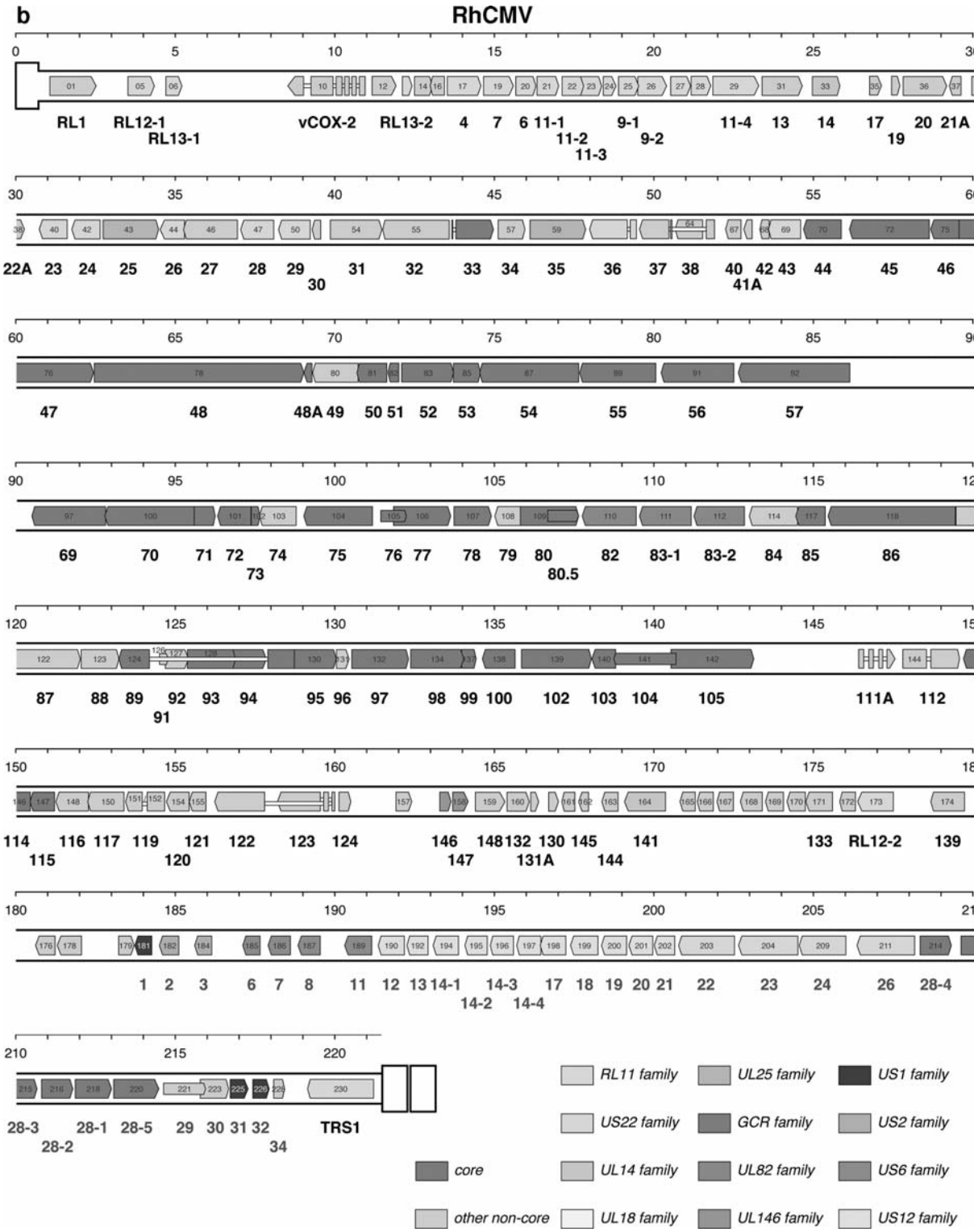
b
RhCMV
RL1 RL12-1 RL13-1 vCOX-2 RL13-2 4 7 6 11-1 11-2 11-3 9-1 9-2 11-4 13 14 17 19 20 21A
22A 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 40 41A 42 43 44 45 46
47 48 48A 49 50 51 52 53 54 55 56 57
69 70 71 72 73 74 75 76 77 78 79 80 80.5 82 83-1 83-2 84 85 86
87 88 89 91 92 93 94 95 96 97 98 99 100 102 103 104 105 111A 112
114 115 116 117 119 120 121 122 123 124 146 147 148 132 131A 130 145 144 141 133 RL12-2 139
1 2 3 6 7 8 11 12 13 14-1 14-2 14-3 14-4 17 18 19 20 21 22 23 24 26 28-4
28-3 28-2 28-1 28-5 29 30 31 32 34 TRS1
RL11 family
UL25 family
US1 family
US22 family
GCR family
US2 family
core
UL14 family
UL82 family
US6 family
other non-core
UL18 family
UL146 family
US12 family

Fig. 59.3. (*cont.*)

Table 59.1. Primate CMV protein identities

ORF	Gene product/function	Identity (%)	
		CCMV/HCMV	RhCMV/HCMV
Herpesvirus core proteins			
UL44	DNA polymerase processivity factor	90	79
UL45	Ribonucleotide reductase	71	59
UL48	Large tegument protein	54	44
UL48A	Smallest tegument protein	63	69
UL54	DNA polymerase	82	74
UL55	Glycoprotein B	76	62
UL56	Transport/capsid assembly	87	82
UL57	Single strand DNA binding protein	77	72
UL70	Helicase/primase component	79	71
UL72	dUTPase	73	61
UL73	Glycoprotein N	52	46
UL75	Glycoprotein H	64	50
UL78	G protein-coupled receptor	60	33
UL80	Assemblin (proteinase)	81	73
UL80.5	Assembly protein precursor	63	50
UL85	Minor capsid protein	86	76
UL86	Major capsid protein	89	78
UL97	Phosphotransferase	71	61
UL98	DNase/exonuclease	87	72
UL99	Tegument protein pp28	50	33
UL100	Glycoprotein M	66	51
UL114	Uracil *N*-glycosylase	83	70
UL115	Glycoprotein L	66	51
Betaherpesvirus-specific proteins			
TRS-1	Tegument protein	63	41
UL25	Tegument protein	60	43
UL27	Unknown	76	58
UL30	Unknown	45	32
UL32	Tegument protein pp150	51	37
UL33	G protein-coupled receptor	71	59
UL36	vMIA	76	48
UL37	vICA	51	32
UL40	NK inhibitor?	52	29
UL74	Glycoprotein O	51	44
UL82	Tegument protein pp71	68	44
UL83	Tegument protein pp65	74	36/42[a]
UL122	Immediate-early 2	72	48
UL123	Immediate-early 1	71	27
US22	Tegument protein	80	47
US24	US22 family	85	66
Primate CMV specific proteins			
UL4	Unknown	25	27
UL18	MHC class I homologue	52	[b]
UL21A	Unknown	69	39
UL111A	vIL-10	[b]	27
UL146	α-chemokine	25	30
US1	Unknown	70	56
US2	Accelerated degradation of MHC class I and II	62	33
US3	Accelerated degradation of MHC class I and II	52	24
US6	Inhibition of TAP	40	26
US11	MHC class I downregulation	47	29
US12	Unknown	58	34
US28/28-5	β-chemokine receptor	71	39

[a]UL83−1 and UL83−2, respectively.

[b]UL18 and 111A homologues not present in RhCMV and CCMV, respectively.

Accession numbers for amino acid identities: BK000394 (HCMV AD169), AF480884 (CCMV), and AY186194 (RhCMV). Amino identities were determined using the GAP program (Scoring Matrix: Blosum62, Gap creation penalty: 8, Gap extension penalty: 2) of SeqWeb® (version 2, San Diego, CA).

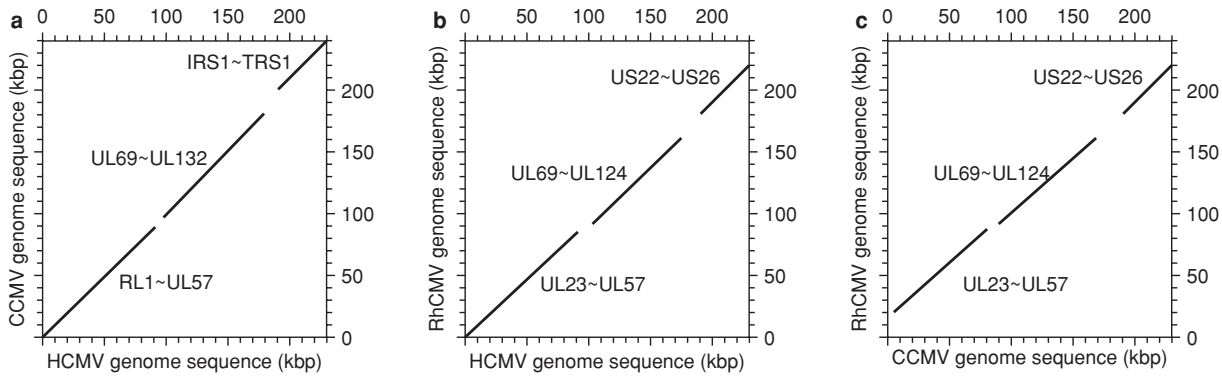

Fig. 59.4. Matrix plot demonstration of the sequence colinearity between (a) HCMV and CCMV, and (b) HCMV and RhCMV (Strain 68–1). The diagrams were computed and plotted using the Compare program (moving window: 30, stringency: 24) of SeqWeb® (version 2, San Diego, CA). The corresponding ORF within the colinear regions of each plot are indicated. The comparison of the HCMV and CCMV genomes using the same criteria has been published previously (Davison *et al.*, 2003). GenBank accession numbers of the genome sequences used for comparison are BK000394 (HCMV AD169), AF480884 (CCMV), and AY186194 (RhCMV).

The conserved gene blocks comprise more than 80% of the total genome content of HHV-6 and HHV-7. The other two betaherpesvirus subgroups are distinguished from HHV-6/7 by larger genome sizes resulting from the presence of highly divergent genes, including tandemly arrayed, genus-specific, gene families. The HCMV genome contains the RL11 family at the left end of the genome and the US2, US6, US12 families near the right end, all of which are present in CCMV and RhCMV (Fig 59.3(a) and 59.3(b)). These families are not present in rodent CMV.

Genomic DNA sequences

Phylogenetic analysis of mammalian and avian herpesviruses based on the sequences of several core-genes, including gB and DNA polymerase, demonstrate that CCMV is the closest known relative of HCMV (McGeoch *et al.*, 2000). Although RhCMV and AGMCMV branch away from the consensus sequence of HCMV and CCMV, these primate CMV cluster into a distinct sublineage among the betaherpesvirus subfamily, taxonomically equivalent to Genus *Cytomegalovirus* (van Regenmortel *et al.*, 2000). Co-speciation of virus and host has been a prominent feature in herpesvirus evolution (McGeoch *et al.*, 1995). The evolutionary branching points that separate the Old from New World primate CMV, and HCMV from CCMV are congruent with the dates of divergence of their hosts (23.3 million years or 5.5 million years ago, respectively) (McGeoch *et al.*, 2000). The DNA sequences of HCMV and CCMV are closely colinear throughout the entire viral genome except for three regions (Fig. 59.4(a)). Regions of divergence include the RL11 gene family locus (7–16 kbp in CCMV) and the origin of DNA replication (*ori*Lyt, 90–98 kbp in CCMV). The largest distinct difference of their sequence organization (180–200 kbp region of CCMV) corresponds to the 19 kbp region that is missing from the AD169 genome but is present in Toledo and clinical isolates (ULb′) (Davison *et al.*, 2003; Cha *et al.*, 1996). The genome sequence of RhCMV exhibits a greater level of sequence discontinuity from HCMV than does CCMV (Figs. 59.4(b)). The colinear relationship of RhCMV to both HCMV and CCMV is largely retained in the central portion of their genomes where the conserved seven core-gene blocks, two beta-gene blocks, and the major immediate-early (MIE) locus are located (Fig. 59.4(b) and 59.4(c)). Sequence discrepancies are found at the *ori*Lyt region (87–90 kbp in RhCMV), the ULb′ region, and the left terminus of the genome. The region of the AGMCMV genome encoding *ori*Lyt (Anders and Punturieri, 1991) is strongly conserved with *ori*Lyt of RhCMV. The sequence from 161 kbp to the right end of the RhCMV genome is no longer colinear to HCMV (Fig. 59.4(b)) or CCMV (Fig. 59.4(c)), except the third beta-gene block comprising the US22-26 ORF. The lack of colinearity in the matrix plots is probably the result of the different copy numbers of ORF belonging to US6, US14, and GCR gene families within this region of RhCMV (Fig. 59.3).

A common feature of the genome in all betaherpesviruses is the absence of any functional coding regions between UL105 and the UL111A ORF. The latter ORF is found only in primate CMV except CCMV (discussed below). As large a genome as CMV possesses, there are relatively few non-coding areas of the genome, outside of the region between UL57 and UL69, which contains *ori*Lyt. The presence of

a 3–4 kbp stretch without apparent coding function begs the question of what function it might serve in the CMV genome, as well as all betaherpesviral genomes. No site-directed mutational analyses have been reported for this portion of the genome, and the area is intact in tissue culture-adapted strains that have deleted other portions of the genome (Cha *et al.*, 1996). Preliminary evidence has been reported that this region of the HCMV genome may represent a stable intron of an unidentified viral transcript (Murphy *et al.*, 2003a, b).

Herpesvirus core proteins

As would be expected for proteins present in all herpesviruses, the core proteins constitute some of the most conserved ORF between HCMV and simian CMV (Table 59.1). Sequence identities of most core proteins are 60%–90% for CCMV/HCMV alignments and 50%–82% for RhCMV/HCMV alignments. The proteins involved in nucleic acid metabolism, DNA replication, and genome packaging are especially conserved when comparing either CCMV or RhCMV with HCMV. The functional importance of the high sequence identity in these proteins is emphasized by in vitro studies evaluating susceptibility to anti-HCMV drugs. RhCMV and HCMV are comparably inhibited by the currently approved compounds ganciclovir, foscarnet (Swanson *et al.*, 1998), and cidofovir, and to benzimidizole nucleosides (North *et al.*, 2004). These drugs are known to target the HCMV DNA polymerase (pUL54), phosphotransferase (pUL97), transport/capsid assembly protein (pUL56), and the DNA packaging protein (pUL89) (Krosky *et al.*, 1998; Underwood *et al.*, 1998; Emery, 2001).

Consistent with the conservation of capsid structure, there is strong sequence conservation of the proteins involved in capsid assembly, particularly for the minor (pUL85) and major (pUL86) capsid proteins mCP and MCP, respectively), and the mCP-binding protein (pUL46). The fourth capsid protein, the small capsid protein (pUL48A), is the most divergent of the group. pUL48A, which has been localized to the hexon tip (Chen *et al.*, 1999), is one of the rare exceptions to the rule that CCMV proteins are more conserved with their HCMV homologues than are those of RhCMV (Table 59.1). The two regions of pUL48A that are the most conserved in any comparison of HCMV, CCMV, and RhCMV (75% identity), corresponding to amino acids 13-31 and 43-75 of HCMV, are involved in binding to the major capsid protein (MCP) of HCMV (Lai and Britt, 2003).

There are five viral proteins, encoded by UL32, 80, 80.5, 82, and 99, that are known to be intimately associated with the capsid either during assembly or within the mature virion, three of which are encoded by core gene members. These include the assemblin maturational proteinase (pUL80) and the capsid assembly protein precursor (pAP) (pUL80.5), both of which are only transiently present within the immature capsid (Chan *et al.*, 2002; Wood *et al.*, 1997), and the tegument phosphoprotein pp28 (ppUL99) (Gibson, 1996). Whereas the proteinase domain of pUL80 is uniformly conserved, the pAP portion exhibits considerably more heterogeneity (Table 59.1). Extensive work with AGMCMV has mapped functional domains within these two proteins. Amino acids involved in the active site and proteolytic cleavage site of assemblin (Welch *et al.*, 1991), and the self-interaction of pAP and binding to MCP (Wood *et al.*, 1997) are the most conserved between simian CMV (AGMCMV, CCMV, and RhCMV) and HCMV. pp28 is the most divergent of all the core proteins with only 50 (CCMV/HCMV) and 33% (RhCMV/HCMV) amino acid identities (Table 59.1). There is only 18% identity between the pp28 proteins of CCMV and RhCMV (not shown). Conserved amino acids in pp28 are predominantly confined to the amino terminal one-third of the protein, suggesting that these may represent the domains that interact with capsid proteins.

Betaherpesvirus-specific proteins

The betaherpesvirus-specific proteins (beta-proteins) have diverged between species to a greater extent than have the core proteins. The CCMV betaherpesvirus proteins are 45%–85% identical with their HCMV equivalents (Table 59.1), which is less than the range of core protein identities (60%–90%). The same proteins of RhCMV have retained 27%–69% amino acid identities with the corresponding HCMV proteins, considerably lower than observed for the core proteins (50%–82%). Sequence variability appears to be limited to comparisons between species and is not a common theme for comparisons of an ORF between isolates of the same species. Some of the RhCMV ORF that are especially divergent from their HCMV counterparts, such as pUL37, ppUL83, and pUL123, are highly conserved between different RhCMV isolates. All three ORF exhibit >97% identity when comparing primary RhCMV isolates with strain 68-1 (these authors, unpublished data). The absence of intraspecies variation and the presence of profound interspecies divergence are consistent with the hypothesis that there were intense selective pressures on these proteins during adaptation to the host. One possible interpretation of the greater divergence of RhCMV beta-proteins versus those of CCMV is that this

group of proteins may interact with cellular proteins to a greater extent than the core proteins. According to this scenario, as the cellular proteins diverged in sequence following the ape/monkey split, there was a compensatory evolution of viral proteins to maintain the putative interactions.

Some of the monkey beta-proteins are almost unrecognizable from their HCMV counterparts. The UL40 protein of HCMV has been implicated in modulation of natural killer (NK) cell activity by virtue of a nine amino acid sequence in its leader peptide that is also found in the leader peptide of most HLA-C molecules (Cerboni *et al.*, 2001). The nonamer peptide of HLA-C is bound by the nonclassical, MHC class I protein, HLA-E, in a TAP-dependent fashion. This complex can activate inhibitory receptors on NK cells. Evidence has been presented that the HCMV-derived peptide of UL40 may function via a similar mechanism, although the issue is not resolved (Cerboni *et al.*, 2001). The RhCMV UL40 protein is only 29% identical to HCMV UL40, and BLASTP searches with it fail to identify its positional homologue within HCMV. However, RhCMV UL40 contains a nonamer sequence within the predicted leader peptide (VMAPRTLLL) that matches that found in the UL40 protein of the AD169 strain and clinical isolates of HCMV (VMAPRTLIL), and CCMV (TMAPKTLLI). One possible implication of the apparent conservation of the UL40 nonamer within the primate CMV UL40 is that dramatic sequence shifts within certain ORF during co-speciation of CMV with its host may not preclude strong functional homology.

Gene duplication events have helped shape the genomes of betaherpesviruses, in general, and CMV, in particular. There are three gene families (UL25, UL82, and US22) within all betaherpesviruses, and 11 unique gene families have been described for HCMV (Davison *et al.*, 2003; Hansen *et al.*, 2003; Chee *et al.*, 1990; Nicholas, 1996; Gompels *et al.*, 1995; Rawlinson *et al.*, 1996; Vink *et al.*, 2000). The HCMV gene families have been conserved both in number and genomic location in both RhCMV and CCMV with one notable exception. The UL82 family is within beta-block II and is comprised of the upper and lower matrix proteins, pp71 (ppUL82) and pp65 (ppUL83), and pUL84. Both RhCMV and BaCMV have a tandem duplication of the UL83 ORF, as opposed to the single UL83 locus found in HCMV and CCMV. This gene duplication produces in a small gap in the matrix plot of the two-dimensional comparison between the RhCMV and either HCMV or CCMV (Fig. 59.4(b) and 59.4(c), around 110–113 kbp in RhCMV). The two RhCMV UL83 ORF are 41% identical at the amino acid level. While the function of pUL83-1 has not been analyzed, pUL83-2 localizes to the nucleus (Yue *et al.*, 2006) and has slightly higher sequence identity to HCMV UL83 than does the RhCMV UL83-1 ORF (42 vs. 36%). In addition, pUL83-2 elicits stronger cellular responses than pUL83-1 (L. Picker, unpublished). The UL83 duplication in monkey CMV bears some resemblance to the situation in murine CMV (MCMV) in which both M83 and M84 loci have sequence homology with HCMV UL83. Although M83 is analogous in position and homologous in sequence to the UL83 gene of HCMV, it also has homology to HCMV pp71 (UL82) (Morello *et al.*, 1999; Cranmer *et al.*, 1996). While the M84 protein is not present within the virion, it, too, has homology with HCMV pUL84, a regulatory protein with incompletely defined functions (Lischka *et al.*, 2003). M84 is considered the MCMV homologue of HCMV pp65 (UL83).

The simian CMV contain two loci found in all other betaherpesviruses that are usually expressed with immediate-early (IE) kinetics, UL36 and 37, and the MIE region coding for IE1 and IE2 (UL123 and 122, respectively) (Fig. 59.3). UL36 and 37 of HCMV code for the viral inhibitor of caspase-8-induced apoptosis (vICA) and viral mitochondrial inhibitor of apoptosis (vMIA), respectively. Both functions are conserved in the RhCMV homologues, although vMIA of RhCMV and HCMV have retained only 32% amino acid identity (McCormick *et al.*, 2003). Interestingly, a naturally occurring RhCMV variant without vICA activity does not impair growth, persistence, or pathogenesis in vivo (Chang and Barry, 2003; Chang *et al.*, 2002; McCormick *et al.*, 2003). Unlike the UL36 and 37 genes in all other betaherpesviruses, which are expressed with IE kinetics, the RhCMV UL36 and 37 genes are transcribed with delayed-early kinetics (McCormick *et al.*, 2003).

The organization, splicing patterns, and strong transcriptional activity of the AGMCMV and RhCMV MIE region (Alcendor *et al.*, 1993; Chang *et al.*, 1990, 1995; Barry *et al.*, 1996; Jeang *et al.*, 1982, 1984, 1987) are similar to those found in HCMV (Stenberg *et al.*, 1989). While the overall structure of the MIE locus is conserved in the betaherpesviruses, there has been significant sequence divergence of the IE coding region in monkey CMV. The IE1 proteins of RhCMV and AGMCMV are only 27%–29% identical with HCMV IE1, the least of all beta-protein comparisons, and only 40% identical with each other (Chang *et al.*, 1995; Barry *et al.*, 1996). In contrast, the IE2 proteins of both monkey CMV are approximately 49% identical with HCMV IE2 and 65% identical to each other (Chang *et al.*, 1995; Barry *et al.*, 1996). The prominent divergence of monkey CMV IE1 is further emphasized by the strong conservation of the CCMV

IE1 and IE2 ORF with those of HCMV (71 and 72%, respectively), suggesting that the monkey CMV IE1 sequence was especially labile during adaptation to a monkey host.

Primate CMV-specific proteins

Primate CMV contain numerous ORF not found in other betaherpesviruses, some of which are restricted to a subset of the primate CMV members. These are largely clustered at either end of the UL portion of the viral chromosome and within the US region, with the exception of the CMV interleukin-10 (cmvIL-10) homologue encoded by UL111A. There are several notable features about these ORF besides their restriction to primate CMV. For the most part, those found within UL are some of the most variable in pairwise alignments between any two primate CMV. Sequence identities range between 25 and 65% for CCMV/HCMV and <29%–39% for RhCMV/HCMV. The left end of UL in RhCMV is so divergent that either the putative ORF within this region (Hansen *et al.*, 2003; Rivailler *et al.*, 2006) are unique to the monkey lineage of CMV and/or only have vestigial homology to their HCMV counterpart, similar to that proposed for UL40. Several of the RhCMV ORF in this region are present within BaCMV, and the DNA sequence of the left end of the RhCMV genome does not align with the corresponding regions of HCMV and CCMV (Fig. 59.4(b) and 59.4(c)).

The right end of UL also has sequence discontinuity between the primate CMV, especially when RhCMV is compared to either HCMV or CCMV. This appears to be the result of a variety of factors including high sequence diversity and the loss of gene colinearity. This same region (UL/b') has either been deleted or undergone rearrangement in tissue culture-adapted strains of HCMV (Cha *et al.*, 1996). The linear order of genes is distinct for each primate CMV (Fig. 59.3). A likely arrangement of ORF within ULb′ has been proposed for RhCMV (Rivailler *et al.*, 2006), and sequence homologues to HCMV UL128, 130, 131A, 132, 148 – 144, and 141 are present within either or both of RhCMV strains 68-1 and 180.92. There are no sequence homologues within RhCMV for HCMV UL140 – 157, although there is a large number of uncharacterized ORF within this region of the RhCMV genome that could possibly be functional homologues. For examples, there are some potential structural similarities between the rh174 ORF of RhCMV with the UL139 ORF of HCMV (these authors, unpublished data). The proposed arrangement of genes within RhCMV ULb′ has been confirmed by sequence amplification of the corresponding region of the RhCMV genome from RhCMV circulating in rhesus macaque populations, with one notable exception. PCR amplification of DNA purified from buccal swabs of naturally infected rhesus macaques has identified an additional 1.5 kb of ULb′ DNA present within wild-type RhCMV not present within either 68-1 or 180.92 (these authors, unpublished data). UL128. The lack of UL128 in RhCMV is intriguing since UL128 has been identified also in the closely related Colburn stain of AGMCMV, albeit with a premature stop codon within the first exon like that in CCMV (Davison *et al.*, 2003; Akter *et al.*, 2003). Some of the genes within the UL/b′ region of simian CMV, such as UL146, have radically diverged from their HCMV homologue to a point of barely detectable homology.

A RhCMV version of the HCMV pUL146 α-chemokine has been identified (accession number AY183378; M. Penfold, unpublished data) based on conservation of a short amino acid sequence (ELRCXC) that is both critical for neutrophil activation by cellular CXC chemokines and present in almost all clinical isolates of HCMV (Prichard *et al.*, 2001). Apart from this motif, however, the remainder of the protein has little identity with HCMV or CCMV pUL146 (30 and 25% amino acid identities, respectively). Notably, the sequence of pUL146 of HCMV is extremely variable (50% divergence) between different clinical isolates (Prichard *et al.*, 2001). UL146 is one of a few HCMV genes with a high degree of intra-species sequence polymorphism (20–50%). Others include UL73 (gN), UL74 (gO), UL144 (TNF receptor), UL4, and UL11 (Rasmussen *et al.*, 2003 and references therein). In contrast, primary isolates of RhCMV from the California National Primate Research Center exhibit 99% identity for pUL146 (these authors, unpublished data).

These ORF appear to be the exceptional sites for genetic heterogeneity between HCMV isolates since most other genes that have been analyzed are considerably more conserved. For example, the US9 and US28 coding regions in clinical isolates of HCMV are >96% identical with the sequences of AD169 (Rasmussen *et al.*, 2003. Therefore, the profound sequence divergence in most of the primate CMV-specific proteins must have occurred during co-speciation with the host following radiation of the primates. The US28 and UL111A ORF, which encode a viral β-chemokine receptor and cmvIL-10 (Lockridge *et al.*, 2000; Kotenko *et al.*, 2000), respectively, are illustrative for the extent of sequence divergence that can occur without loss of functional activity.

RhCMV, BaCMV, and AGMCMV (strain 9610, accession numbers AY340790–340794) (Sahagun-Ruiz *et al.*, 2004) each contain a tandem array of five genes that are homologous to HCMV US28 by position and sequence. The five

RhCMV US28 proteins have a range of identities with HCMV pUS28 (24–39%) that is comparable to the identities between HCMV pUS27 and pUS28 (31%) and between themselves (21%–41%). Of the five, only one (RhCMV US28-5) binds the membrane-bound chemokine, fractalkine (FKN), and multiple β-chemokines with similar binding affinities as HCMV pUS28 (Penfold *et al.*, 2003). Moreover, functional RhCMV US28-5 has been localized to the mature virion, like HCMV US28 (Penfold *et al.*, 2003). Based on the low homology between RhCMV pUS28-5 and HCMV pUS28 (39% identity), it is reasonable to speculate that evolution of the primate US28 coding regions has been driven by maintenance of a high affinity for the host's chemokines. However, it appears that the US28 chemokine receptors have diverged to a much greater extent than might be predicted by the rate of divergence of cellular ligands. The human and rhesus macaque FKN proteins are 93% identical, indicating that US28 evolution was not proportionate to the rate of change of this particular cellular ligand. The evolutionary pressures that have driven the rate of change of pUS28 sequences may be especially complicated since pUS28 binds to multiple cellular ligands (Penfold *et al.*, 2003).

Similarly, cmvIL-10 proteins encoded by RhCMV and HCMV are as divergent from each other (27% identical) as they are from their host species' cellular IL-10 (cIL-10) (Lockridge *et al.*, 2000). The divergence of cmvIL-10 is distinct from viral IL-10 encoded by EBV (ebvIL-10), which is 90% identical to cIL-10. The sequence of cmvIL-10 is stable amongst multiple clinical isolates of HCMV (Hector and Davison, 2003) and RhCMV (these authors, unpublished data), indicating that sequence variation occurs between species, not within species. cmvIL-10 functions through the IL-10 receptor (Kotenko *et al.*, 2000; Jones *et al.*, 2002; Spencer *et al.*, 2002), binding with an affinity almost identical to that of cIL-10 (Jones *et al.*, 2002). As with pUS28, it appears that the cmvIL-10 proteins have diverged to a much greater extent than might be predicted by the rate of divergence of cIL-10 receptors. Although there is no available monkey IL-10 receptor sequence to enable a rhesus/human comparison, rhesus dendritic cells (DC) respond as well as human DC to recombinant human cIL-10 (these authors, unpublished data), suggesting a strong conservation of the IL-10 receptor between humans and rhesus macaques. Transduction of cIL-10 by a progenitor primate CMV and subsequent genetic drift in sequence could impart new functions to the viral IL-10, although this presents something of a conundrum for the virus. On one hand, genetic drift in a transduced cellular gene can result in the acquisition of novel functions. On the other, this could result in the conversion of a non-immunogenic "self" protein into a highly immunogenic "non-self" protein. The crystal structure of the cmvIL-10 homodimer bound to the high affinity IL-10 receptor is similar to that of cIL-10 and the receptor (Jones *et al.*, 2002). Distinctions in quaternary structures between these two complexes suggest potential differences in downstream signaling pathways, although it has not yet been determined what functional phenotype separates cmvIL-10 from cIL-10 (Kotenko *et al.*, 2000; Jones *et al.*, 2002; Spencer *et al.*, 2002).

The UL111A locus of primate CMV is in a state of genetic flux. The structure of the cIL-10 gene consists of five exons/four introns. The monkey UL111A genes (RhCMV, AGMCMV, and BaCMV) have lost the intron between exons 4 and 5 (126), while the HCMV UL111A has further lost the intron between exons 3 and 4 (Lockridge *et al.*, 2000; Kotenko *et al.*, 2000). In contrast, the ebvIL-10 gene has no introns. CCMV has no UL111A ORF (Davison *et al.*, 2003), similar to the rodent CMV genomes. It may be that CCMV, like murine CMV, up-regulates expression of cellular IL-10 following infection of cells (Redpath *et al.*, 1999), obviating the need to both encode its own IL-10 function and maintain the integrity of the UL111A gene.

The absence of UL111A in CCMV (and rodent CMV) serves as an important reminder that while the primate CMV share an overwhelming repertoire of genes and similarities in natural history, each species has adapted to its niche in its own unique way, jettisoning, transducing, or modifying coding capacity and regulatory regions as selective pressures dictated. In some cases, this meant having to evolve independent mechanisms for similar virus/host scenarios, as may be the case for activation of IL-10 through expression in the context of either the viral or host genome. Another potential example of this is provided by the apparent importance of cyclooxygenase 2 (COX-2) in the CMV life cycle.

Infection of human fibroblasts with HCMV alters the level of more than 250 cellular genes prior to replication of the viral genome, including COX-2 (Zhu *et al.*, 1998). The function of elevated COX-2 remains to be determined, but the constellation of cellular genes whose expression levels are temporally changed along with COX-2 point to a need for the virus to regulate intracellular and extracellular inflammatory responses. Indeed, HCMV infection of fibroblasts is severely attenuated in the presence of COX-2 inhibitors (Zhu *et al.*, 2002). RhCMV does not increase cellular COX-2 levels (J. Nelson, unpublished data), but it may have come up with its own solution to the regulation of COX-2. RhCMV encodes a multiply spliced COX-2 homologue at the left end of UL that is approximately 75% identical with the cellular COX-2 protein (Hansen *et al.*, 2003).

Deletion of RhCMV COX-2 does not impair growth in fibroblasts but does inhibit replication in endothelial cells (J. Nelson, unpublished data). Assuming retention of function, transduction of cellular COX-2 by monkey CMV would appear to be an example of convergent evolution whereby the virus is better able to control its own destiny by modulating host inflammatory processes. Although the mechanism may differ from that employed by HCMV, the net effect is probably the same.

Summary

In sum, the simian CMV are an important primate complement to the other animal models of HCMV. Progress in characterizing them at the molecular and biological levels facilitates new avenues of inquiry into the mechanisms of HCMV persistence and pathogenesis. It is now possible to define the role of specific gene products in CMV natural history by applying the same DNA modification tools elegantly used for genetic engineering of bacterial artificial chromosomes containing the genomes for HCMV, MCMV, RCMV, and GPCMV. The full-length RhCMV genome has been cloned into a BAC (Chang *et al.*, 2003), enabling the efficient and accurate introduction of any site-directed mutation into the viral genome. In addition, novel vaccine and chemotherapeutic intervention strategies can be tested in primate hosts that closely approximate the human condition.

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Gammaherpesviruses of New World primates

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Introduction

Numerous Gamma-herpesviruses, a large subfamily of the herpes group, have limited pathogenic potential upon primary infection of their natural host. They are most relevant however as tumor viruses of the hematopoietic system and form an important chapter of viral oncology. The prototype of the genus lymphocryptovirus (γ1-herpesvirus), Epstein-Barr Virus (EBV), was the first clearly identified human herpesvirus. EBV causes lymphomas of B-cell origin and other lymphoproliferative syndromes, nasopharyngeal carcinomas and, possibly, gastric cancer. The second known genus of gamma-herpesviruses, rhadinoviruses or γ2-herpesviruses, is biologically and molecularly distinct. The prototypic members of this group, termed Herpesvirus (H.) saimiri (HVS) and H. ateles (HVA), were detected as T-lymphotropic tumor viruses in neotropical primates and raised primary interest from the fact that they cause fulminant T-cell lymphomas in numerous primates as well as in rabbits, although no exact correlates of these tumors exist in human pathology. This led to the identification of novel viral membrane-associated T-cell oncoproteins, termed Stp and Tip. These are small adaptor molecules that efficiently act on T-lymphocyte signaling. The viruses have been used as expression vectors in T-lymphocytes and allow to study mechanisms of episomal persistence in components of the T-cell system. Later on it became clear that certain strains of HVS can transform human T-lymphocytes to continuous growth in an antigen- and mitogen-independent fashion, providing for the first time a reliable means of human T-lymphocyte immortalization in cell culture. Additional interest in the γ2-herpesviruses arose when the Kaposi-sarcoma-associated Human Herpesvirus 8 (KSHV/HHV8) was identified as the first human pathogenic rhadinovirus. However, in view of the complex biology of Kaposi's Sarcoma or other neoplastic diseases such as body cavity-based B-cell lymphomas and multifocal Castleman's Disease, it is by far less clear which KSHV/HHV8 genes encode the relevant oncoproteins of KSHV/HHV-8. In this chapter, we wanted to focus mostly on the basic biology and gene expression profiles of HVS and HVA, on the viral mechanisms of oncogenic transformation, and possible applications of these viruses as T-cell vectors and in cell-based immunotherapy.

Herpesvirus saimiri

Natural occurrence and pathology

The rhadinovirus (γ2-herpesvirus) Herpesvirus saimiri (HVS) is regularly found in squirrel monkeys (*Saimiri sciureus*), whose natural habitat are South American rainforests. Squirrel monkeys are usually infected via saliva within the first two years of life. The virus does not cause disease or tumors and establishes lifelong persistence in the species (Melendez *et al.*, 1968). In other New World primate species such as tamarins (*Saguinus (S.) spp.*), common marmosets (*Callithrix (C.) jacchus*) or owl monkeys (*Aotus trivirgatus*), the infection with HVS causes acute peripheral T-cell lymphoma within less than two months after experimental infection (Melendez *et al.*, 1969; Wright *et al.*, 1976; for review see Fleckenstein and Desrosiers, 1982). The experimental infection is usually performed intramuscularly or intravenously. Intramuscular injection of purified virion DNA also causes disease in susceptible primates (Fleckenstein *et al.*, 1978b). HVS strains were classified into the three subgroups A, B and C depending on the pathogenic properties and on the sequence divergence in the left-terminal non-repetitive genomic

region (Desrosiers and Falk, 1982; Medveczky *et al.*, 1989; Medveczky *et al.*, 1984). The major representative strains are A11 (Falk *et al.*, 1972) for subgroup A, B-S295C (Melendez *et al.*, 1968) and B-SMHI (Daniel *et al.*, 1975) for subgroup B, and C488 (Biesinger *et al.*, 1990) and C484 (Medveczky *et al.*, 1984) for subgroup C. Viruses of subgroup B are considered to be less oncogenic, subgroup C strains have the strongest oncogenic properties. Tamarins are susceptible to viruses of all subgroups, while subgroup B viruses were reported as not being able to cause disease in adult common marmosets (reviewed in Fickenscher and Fleckenstein, 2002). The parental strain C488 as well as various viral deletion mutants cause acute peripheral T-cell lymphoma within only a few weeks in common marmosets or in cottontop tamarins (*S. oedipus*) (Duboise *et al.*, 1998a; Ensser *et al.*, 2001; Glykofrydes *et al.*, 2000; Duboise *et al.*, 1998b; Knappe *et al.*, 1998a,b). Remarkably, high intravenous doses of HVS strain C488 can induce a similar fulminant disease in Old World monkeys such as rhesus and cynomolgus monkeys (*Macaca (M.) mulatta, M. fascicularis*). The pathological findings in macaques were similar to those in New World primates, and the disease in cynomolgus monkeys was designated as a pleomorphic peripheral T-cell lymphoma or alternatively as a pleomorphic T-lymphoproliferative disorder (Alexander *et al.*, 1997; Knappe *et al.*, 2000a). Whereas HVS infection or pathogenicity has not been reported in rodents, a non-permissive infection and tumor induction was described in New Zealand white rabbits, although with variable efficiency (Ablashi *et al.*, 1985; Medveczky *et al.*, 1989). HVS can be isolated from peripheral blood cells of persistently infected squirrel monkeys, or from leukemic animals, presumably from infected T cells, by co-cultivation with permissive owl monkey kidney (OMK) cells (Falk *et al.*, 1972). HVS replicates productively and induces cell lysis of OMK cells (Daniel *et al.*, 1976). A series of transformed T-cell lines were derived from leukemias or tumors of virus-infected tamarins and could be cultivated continuously for several years (reviewed in Fleckenstein and Desrosiers, 1982). While virus particles were found initially in most cases, virus production was frequently lost after prolonged culture. The episomal DNA is heavily methylated in such cell lines (Desrosiers *et al.*, 1979), and some of these cell lines carried rearrangements or large deletions in the episomal HVS genomes (Kaschka-Dierich *et al.*, 1982). Marmoset and tamarin T-cells can be transformed by HVS to stable T-cell lines in vitro and are designated as semi-permissive, since virus particles are released, although to lower titers than from OMK cells (Desrosiers *et al.*, 1986; Kiyotaki *et al.*, 1986; Schirm *et al.*, 1984; Szomolanyi *et al.*, 1987).

Genome structure and replication

HVS is the prototype of the subfamily *rhadinovirus* (g2-herpesviruses) (Roizman *et al.*, 1992). The term "rhadino" viruses uses the ancient Greek word "ραδιυοσ" for fragile (Roizman *et al.*, 1992), because the genomic viral DNA splits upon isopyknic centrifugation into two classes of highly different density, the L-DNA containing the viral protein-coding genes (low density, low G+C content) and the terminal non-coding repetitive H-DNA (high density, high G+C content). The intact rhadinoviral so-called M-genome has intermediate density in CsCl gradients (M-DNA). Two strains of HVS, #A11 and the highly oncogenic subgroup C strain #C488 were sequenced (Albrecht *et al.*, 1992a; Ensser *et al.*, 2003). In the case of HVS A11, the H-DNA contains multiple tandem repeats of 1444 bp with 70.8% G+C, whereas the long unique L-DNA has 112 930 bp with 34.5% G+C (Albrecht *et al.*, 1992a; Fleckenstein and Desrosiers, 1982). The size of the total M-DNA genome is variable due to different numbers of H-DNA segments attached to both ends of the linear virion genome. In strain C488, the L-DNA comprises 113027 bp, and it is flanked by two distinct repeat units of 1318 and 1458 bp. The shorter unit is a subset of the longer repeat unit of which 140 bp are deleted. The size of the packaged C488 M-genome is approx. 155 kbp, ranging from 130–160 kbp due to variable numbers of terminal H-DNA segments (Ensser *et al.*, 2003). The HVS L-DNA genomes contain at least 76 to 77 open reading frames and 5 to 7 U-RNAs (Albrecht *et al.*, 1992a; Hör *et al.*, 2001; Ensser *et al.*, 2003). These are the gene blocks of typical herpesvirus genes which are highly conserved between herpesvirus families (Gompels *et al.*, 1988; Albrecht and Fleckenstein, 1990). Flanking or interspersed are genes which do not usually occur in other herpesviruses families, among these are transforming oncogenes and viral homologues of cellular genes which will be described below. While most genes are well conserved between different HVS strains, there is extensive sequence variation at the so-called left end of the HVS L-DNA and in the region of the R transactivator gene *orf50* and the glycoprotein gene *orf51* (Biesinger *et al.*, 1990; Thurau *et al.*, 2000; Hör *et al.*, 2001; Ensser *et al.*, 2003). The genome structures of HVS and KSHV/HHV-8 are compared in Fig. 60.1. Not much is known about the replication mechanisms of rhadinoviruses in general or of HVS in particular. The HVS A11 origin of lytic replication was mapped to the untranslated region upstream of the thymidylate synthase gene (Lang and Fleckenstein, 1990; Schofield, 1994). A putative origin in the left-terminal region of the L-DNA was described to mediate plasmid maintenance in strain C484 (Kung and Medveczky, 1996) that is neither conserved between

different HVS strains, nor is it required for viral replication or episomal persistence in strain C488 or C484 (Ensser *et al.*, 1999; Medveczky *et al.*, 1989). In transformed human T cells, HVS persists as stable non-integrated episomes at high copy number (Biesinger *et al.*, 1992). There are no indications yet for the genetic correlate of a plasmid-like origin of replication and of the viral factors involved. In contrast to herpes simplex virus, the infection of tissue culture cells by HVS is asynchronous (Randall *et al.*, 1985). Therefore, the classification of HVS genes to the immediate-early (IE) phase of infection has been difficult and was mostly based on experiments using cycloheximide to inhibit viral protein synthesis. The IE gene *ie57* codes for a nuclear phosphoprotein of 52 kDa (Nicholas *et al.*, 1988; Randall *et al.*, 1984) with structural and functional homology with ICP27/IE63 of herpes simplex virus and EBV BMLF1. Correspondingly, IE57 stimulates the expression of unspliced and represses the expression of spliced transcripts (Whitehouse *et al.*, 1998). IE57 further redistributes nuclear components of the splicing machinery (Cooper *et al.*, 1999) and is involved in nuclear RNA export (Goodwin *et al.*, 1999). Thus, the *ie57* post-transcriptional regulator appears to be the sole regulatory viral IE gene. A strong viral transactivator function was mapped to the delayed-early gene *orf50* (Nicholas *et al.*, 1991), the homologue to the R transactivator of EBV. Due to differential splicing and promoter usage, the gene codes for a larger protein ORF 50A and for a smaller C-terminal variant ORF 50B. The transactivation domain resides in the C-terminus of the ORF 50 proteins and binds to the TATA-binding protein in the basal transcription complex. Post-transcriptional inhibition of spliced *orf50A* transcripts by the IE57 can not have functional relevance in this context, suggesting that orf50 exerts the dominant function for regulation of replication, at least in HVS strain C488. While *ie57* is highly conserved between subgroup A and C, the genomic *orf50* region encoding this major viral transactivator was found to be strongly divergent (Thurau *et al.*, 2000; Ensser *et al.*, 2003). Neither HVS nor HVA encode a homologue to the bZip/Zta of EBV or KSHV (Sinclair, 2003). The HVS ORF73 protein of strain A11 and C488 localizes to the host cell nucleus, and like the latent nuclear antigen LANA of KSHV, it can associate with host cell chromosomal DNA (Hall *et al.*, 2000; Schäfer *et al.*, 2003). The A11 ORF73 is highly expressed in HVS-infected human epithelial cancer cell lines, it can associate with the cellular p32, thereby coactivating heterologous as well as its own promoter (Hall *et al.*, 2002). A11 ORF73 further binds to GSK-3ß, which is involved in *WNT*-signaling and possibly contributes to oncogenic transformation (Fujimuro and Hayward, 2003). Although not detectable by Northern blotting from C488-transformed human T-cells (Fickenscher *et al.*, 1996), transcripts of this gene are found by RT-PCR. The HVS C488 orf73 gene product can downregulate the orf50A and B promoters and prevents the ORF50 mediated activation of viral replication gene promoters, and it can block the initiation of the lytic replication cascade. This suggests that HVS ORF73 can control the transition between rhadinoviral latency and lytic replication (Schäfer *et al.*, 2003).

Rhadinoviruses like HVS and KSHV contain several intronless viral genes that are homologous to cellular genes; it might be speculated on a role for reverse transcription in this context. While a few of these likely captured cellular gene homologs are unique to specific viruses, some are common to several rhadinoviruses (Fig. 60.1) or to γ-herpesviruses including EBV. This may suggest that the uptake of cellular genes is a rather infrequent event during herpesvirus evolution. Most of these cellular homologues can be categorized into two major groups: (I) genes related to nucleotide metabolism or cellular growth control, and (II) genes that modulate innate or adaptive immune functions (discussed below). HVS homologues to enzymes of the nucleotide metabolism include a dihydrofolate reductase (*orf2*, DHFR) and a functional thymidylate synthase (*orf70*, TS). Both *orf3* and *orf75* encode large tegument proteins, which share local homology to formylglycineamide ribotide amidotransferase (FGARAT) (summarized in Ensser *et al.*, 2003). The functions of these enzymes may possibly augment the free nucleotide pool and could thus facilitate DNA synthesis and virus replication. *Orf2* and the adjacently located viral U-RNA genes are dispensable for virus replication and T-cell transformation (Ensser *et al.*, 1999). HVS *orf72* codes for a functional viral cyclin D that is expressed in semipermissive HVS-transformed marmoset T-cells and is resistant to cyclin-dependent kinase inhibitors $p16^{Ink4a}$, $p21^{Cip1}$ and $p27^{Kip1}$. This deregulation pushes the cell cycle towards the S phase, thereby supporting virus replication in permissive cells (Nicholas *et al.*, 1992; Jung *et al.*, 1994; Swanton *et al.*, 1997). Although enhanced replication might secondarily promote transformation or tumor induction, the viral cyclin seems to provide only auxiliary functions, since it is not required for replication, T-cell transformation and lymphomagenesis (Ensser *et al.*, 2001).

Immunomodulatory proteins

Rhadinoviruses target various host mechanisms involved in pathogen elimination, both in the innate and adaptive immune system. Two HVS genes are functional regulators of the complement system; the *orf4* codes for a complement control protein homologue (CCPH), which inhibits C3 convertase, an enzyme involved in the initiation of early steps in

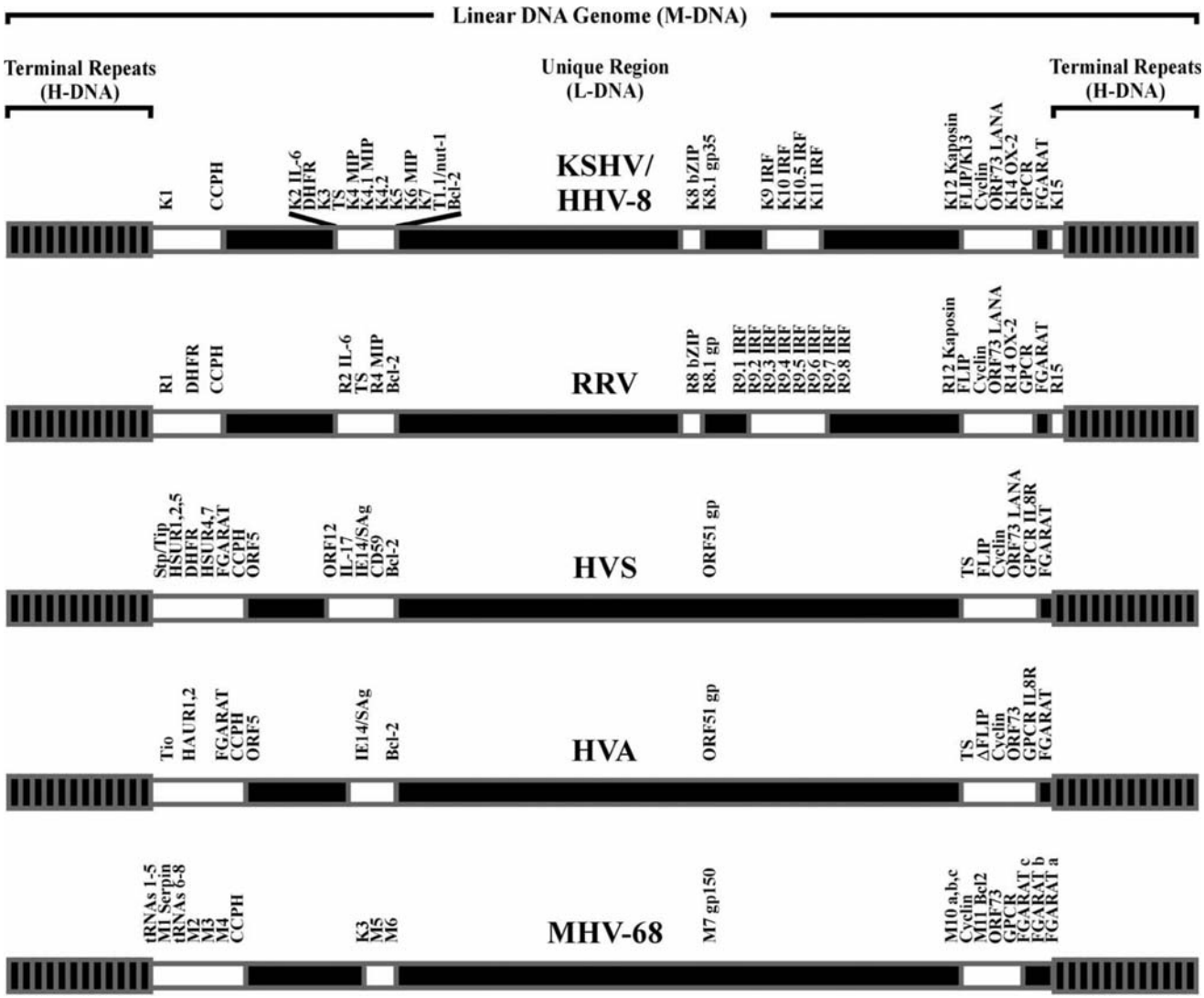

Fig. 60.1. Genome structure of selected rhadinoviruses. The genome structures of the rhadinoviruses KSHV/HHV-8 (Russo *et al.*, 1996; Neipel *et al.*, 1997), rhesus rhadinovirus (RRV; Alexander *et al.*, 2000; Searles *et al.*, 1999), herpesvirus saimiri (HVS; Albrecht *et al.*, 1992a; Ensser *et al.*, 2003), herpesvirus ateles (HVA; Albrecht, 2000), murine herpesvirus 68 (MHV-68; Virgin *et al.*, 1997) are shown with special respect to variable areas harboring non-conserved genes or genes with homology to cellular counterparts (white boxes). Conserved genomic regions of virus genes with typical herpesvirus functions are shown in black. Abbreviations are: bZIP, basic-leucine zipper protein. CCPH, complement control protein homologue. DHFR, dihydrofolate reductase. FGARAT, formylglycineamide ribotide amidotransferase. FLIP, FLICE inhibitory protein. gp, glycoprotein. GPCR, G-protein coupled receptor. HSUR or HAUR, HVS or HVA-encoded URNA. IRF, interferon regulatory factor. MIP, macrophage-inflammatory protein. SAg, superantigen homologue. Stp, saimiri transformation-associated protein. Tio, two-in-one-protein. Tip, tyrosine kinase-interacting protein. TS, thymidylate synthase.

complement activation (Albrecht and Fleckenstein, 1992; Fodor *et al.*, 1995). *Orf15* is a viral variant of CD59, which prevents the insertion of the membrane attack complex formed by C8 and C9, and thus blocks the terminal complement cascade (Albrecht *et al.*, 1992b; Rother *et al.*, 1994). T-cell stimulatory functions have been described for cellular CD59 via CD2 (Korty *et al.*, 1991; Deckert *et al.*, 1995), but functional data are not available for the viral CD59. The orf12 has homology to the K3 and K5 genes of KSHV (Coscoy and Ganem, 2000; Ishido *et al.*, 2000a,b; Means *et al.*, 2002), but is dispensable for replication and human T-cell transformation in vitro (Knappe *et al.*, 1998a).

Several rhadinoviral ORFs encode potent inhibitors of cell death or apoptosis, such as *orf16* and *orf71*. The ORF16 is a viral Bcl-2 homologue (Bellows *et al.*, 2000; Derfuss *et al.*, 1998; Nava *et al.*, 1997). Both Bcl-XL and viral Bcl-2 inhibit cell death induced by either cell-autonomous (independent of death receptors) or receptor-mediated mechanisms, depending on the cell type studied. The ORF71, a viral FLICE (FADD-like interleukin 1-converting enzyme (ICE)-like protease) inhibitory protein (vFLIP), interacts with cellular FADD (Fas-associated protein with death domains) and FLICE via homophilic interaction of their respective death-effector domains, therefore blocks formation of the death-signal-induced signaling complex, and consequently prevents caspase 8 (FLICE) activation. Although the vFLIP inhibited death-receptor-dependent apoptosis and partially protected permissive OMK cells from Fas-dependent apoptosis at a late stage of infection (Thome *et al.*, 1997), the vFLIP was dispensable for virus replication to high virus titers, T-cell transformation and lymphoma induction (Glykofrydes *et al.*, 2000).

The HVS gene *orf13* led to the discovery of its cellular homologue ctla-8 (Rouvier *et al.*, 1993) that codes for IL-17, a CD4+ T-cell specific cytokine. The viral IL-17 is functionally not distinguishable from its cellular counterpart that is capable of supporting T-cell proliferation (Yao *et al.*, 1995; Fossiez *et al.*, 1998). However, deletions of the HVS C488 *orf13*/*vIL-17* had no phenotype with regard to virus replication and oncogenicity (Knappe *et al.*, 1998a). In contrast to the vIL17 that is unique to HVS, G-protein coupled receptors (GPCR) are found in most rhadinoviruses. The HVS *orf74* encodes a viral IL-8 receptor (IL-8R) that is classified to the low-affinity B type of IL-8R (Ahuja and Murphy, 1993; Murphy, 1994; Nicholas *et al.*, 1992).

The viral immediate early gene *orf14*/*vSag* has local homology to the superantigen (Sag) of mouse mammary tumor virus (MMTV) and to murine *mls* superantigens (Thomson and Nicholas, 1991). Although recombinant viral IE14/vSag protein bound to MHC class II molecules and stimulated T-cell proliferation, there is no evidence of a selective advantage for specific Vb families that would be typical for superantigens, neither after stimulation of human T-cells with IE14/vSag in vitro (Yao *et al.*, 1996; Duboise *et al.*, 1998a), nor after infection and transformation with HVS (Knappe *et al.*, 1997). The HVS C488 ie14/vsag (Thomson and Nicholas, 1991) is dispensable for viral replication but its role in the transformation of human and simian T-cells in vitro or pathogenicity has remained controversial (Knappe *et al.*, 1997, 1998b; Duboise *et al.*, 1998a). Like other rhadinoviruses, HVS has acquired a series of cellular genes, but most if not all of these genes are dispensable for virus replication, T-cell transformation in culture, and pathogenesis in susceptible New World primates. Similar to KSHV infected B-cell lines, many of the cell-homologous viral genes are only expressed during lytic virus replication, but not in transformed human lymphocytes (Fickenscher *et al.*, 1996; Knappe *et al.*, 1997).

Oncogenesis

The major factors responsible for induction of T-cell leukemia and T-cell transformation in vitro reside in the variable region at the left end of the HVS L-DNA (Koomey *et al.*, 1984; Desrosiers *et al.*, 1985a; Desrosiers *et al.*, 1986; Murthy *et al.*, 1989; Chou *et al.*, 1995; Duboise *et al.*, 1998b). In subgroup A and B strains there is only one gene at this position, termed stpA or stpB (saimiri transformation associated protein of subgroup A or B strains) (Murthy *et al.*, 1989; Hör *et al.*, 2001). At the homologous location, the virus strains of subgroup C carry two open reading frames, that were later termed *stpC* (saimiri transformation-associated protein of subgroup C strains) and *tip* (tyrosine kinase interacting protein) (Biesinger *et al.*, 1990; Jung and Desrosiers, 1991; Biesinger *et al.*, 1995) (Fig. 60.2). The closely related HVA encodes the protein Tio at the homologous genomic position (Albrecht *et al.*, 1999). StpA and B share limited sequence homology with StpC, but are structurally unrelated to Tip (Fig. 60.2). While not required for viral replication, deletion of either *stpC* or *tip* abolishes the transformation by HVS in vitro and pathogenicity in vivo (Duboise *et al.*, 1996, 1998b; Knappe *et al.*, 1997; Medveczky *et al.*, 1993). Both *stpC* and *tip* genes are transcribed into a single bicistronic mRNA from a common promoter directed toward the left genomic end of the L-DNA, where *tip* is situated downstream of *stpC*. The transcription of *stpC*/*tip* is regulated similarly to cellular IE genes in human T-cells and no obvious viral factors seem to be involved. In C488-transformed human T-cells, *stpC* and *tip* are the only constitutively transcribed viral genes. (Biesinger *et al.*, 1995; Fickenscher *et al.*, 1996; Fickenscher *et al.*, 1997). The StpC protein is a 102 aa perinuclear membrane-associated phosphoprotein with a predicted molecular mass of approximately 10 kDa but migrates with an apparent molecular mass of 20 kDa. The N-terminus of StpC consists of 17 mostly charged amino acids. The C-terminus is a hydrophobic region, which probably serves as an anchor to perinuclear membranes. In between are 18 collagen tripeptide repeats (GPX)n, which may mediate multimerization of the protein (Fig. 60.2) (Biesinger *et al.*, 1990; Fickenscher *et al.*, 1997; Jung and Desrosiers, 1991, 1992, 1994). *stpA* of strain A11 and *stpC* of strain C488 transfected rodent fibroblasts formed foci in vitro and induced tumors in nude mice (Jung *et al.*,

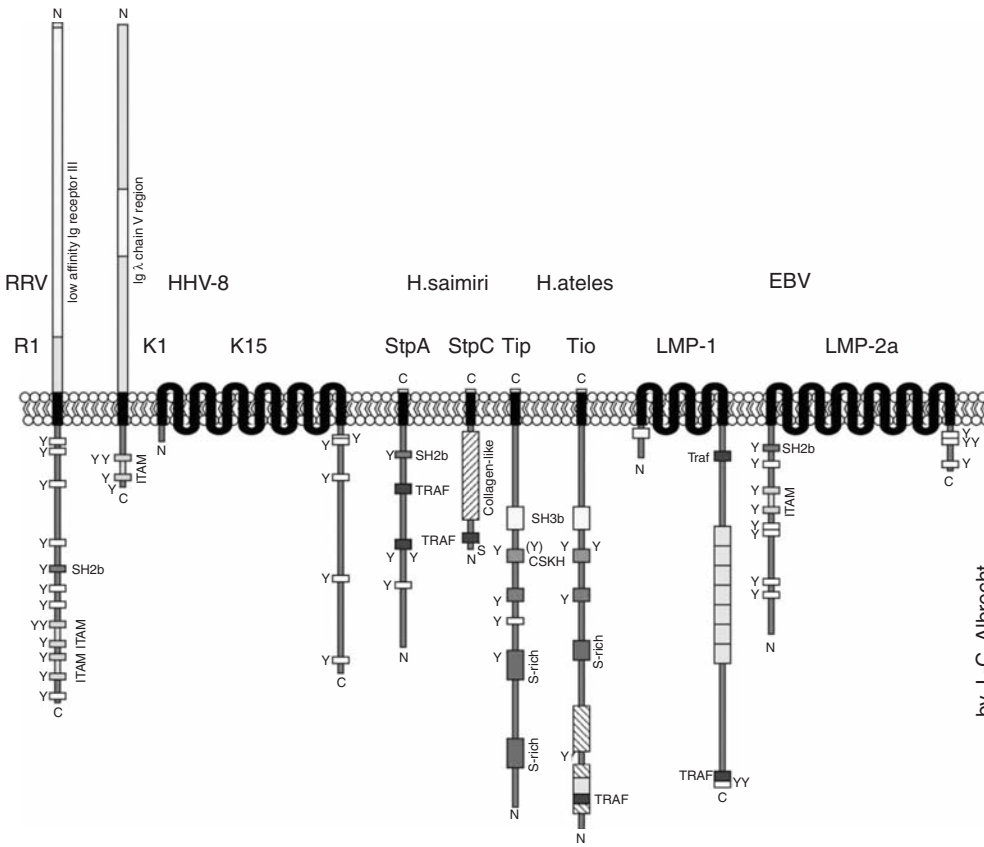

Fig. 60.2. Structural diversity of the major gammaherpesvirus signaling proteins. The HVS oncoproteins Stp, Tip, or HVA Tio, the KSHV K1 and K15, RRV R1 proteins are shown along with the EBV LMP1, LMP2a. CSKH, C-terminal Src kinase homology region. ITAM, immunoreceptor tyrosine-containing activation motif. SH3b, domain interacting with src-family kinase SH3 domain. SH2b, domain interacting with src-family kinase SH2 domain. S-rich, Serine rich motif. TRAF, potential binding site for TNF-R associated factors. Y and YY, tyrosine and double-tyrosine residues, putative signaling motifs. Black, Transmembrane domain. N or C, amino- or carboxyterminus of the protein, respectively.

1991). *stpA* transgenic mice developed polyclonal peripheral T-cell lymphoma, while an *stpC* transgene induced epithelial tumors (Kretschmer *et al.*, 1996; Murphy *et al.*, 1994). StpA was reported to bind to and to be phosphorylated by the non-receptor tyrosine kinase Src (Lee *et al.*, 1997). The non-transforming StpB also associates with Src (Choi *et al.*, 2000). StpC was shown to interact with the small G-protein Ras and stimulated mitogen activated protein (MAP) kinase activity (Jung and Desrosiers, 1995). Both proteins StpA and StpC interact with TNF-associated factors (TRAFs) leading to nuclear factor kappa B (NFkB) activation (Lee *et al.*, 1999; Sorokina *et al.*, 2004). Tip was first observed as a 40 kD phosphoprotein that coprecipitated with the T-cell-specific non-receptor tyrosine kinase $p56^{lck}$ in C488-transformed T-cells and was only detectable by a phosphotransferase assay. The protein was thus named tyrosine kinase interacting protein (Tip) and could be identified as the gene product of the leftmost C488 reading frame (Biesinger *et al.*, 1995). Tip of HVS strain C488 has 256 aa and a predicted molecular mass of 29 kD. Considerable interstrain variation of the *tip* genes has been observed (Greve *et al.*, 2001; Ensser *et al.*, 2003), but the common denominators are a N-terminal glutamate-rich region, which is duplicated in some strains, followed by one or two serine-rich regions, a bipartite kinase interacting domain, and a C-terminal hydrophobic domain which anchors the molecule at the inside of the plasma membrane. The kinase interacting domain consists of nine amino acids with homology to the C-terminal regulatory regions of various Src kinases (CSKH), and a proline-rich SH3-domain-binding sequence (SH3B); both motifs are required for the interaction with the kinase (Fig. 60.2) (Biesinger *et al.*,

1995; Jung *et al.*, 1995a; Heck *et al.*, 2006). Several tyrosine residues, three of which are conserved between all strains investigated, are a substrate for the tyrosine kinase Lck. The phosphorylated tyrosine residue 127 of Tip (pY127) was identified by biophysical binding analyses as a potential third Lck interaction site at the Lck-SH2 domain (Bauer *et al.*, 2004). Y127 is the major tyrosine phosphorylation site of Tip, but this does not enhance Lck binding in T cells. Recombinant virus expressing mutations in Tip show that the strong Lck binding mediated by cooperation of both SH3B and CSKH motifs is essential for transformation of human T cells by herpesvirus saimiri C488, whereas the Y127 of Tip was particularly required for transformation in the absence of exogenous interleukin-2, suggesting its involvement in cytokine signaling pathways (Heck *et al.*, 2006). Tip binding to Lck modulates the kinase activity: (Fickenscher *et al.*, 1997; Hartley *et al.*, 1999; Lund *et al.*, 1997a; Wiese *et al.*, 1996). When compared with their non-infected parental clones, HVS C488-transformed human T-cell clones had increased basal levels of tyrosine phosphorylation (Wiese *et al.*, 1996). Dependent on the presence of both the CSKH or SH3B motifs, Tip could activate Lck even when the regulatory tyrosines Y394 and Y505 of Lck had been mutated, suggesting a novel mechanism of Lck activation (Hartley *et al.*, 1999), which may result in an altered substrate specificity of the kinase contributing to the abrogation of ZAP70 phosphorylation (Cho *et al.*, 2004). This dysregulation may further link Tip-bound Lck to alternative downstream effectors. Since constitutively active Lck mutants have an oncogenic potential, stimulation of Lck signaling by Tip would contribute to the activated phenotype of HVS-transformed T-cells and to the transformation process, whereby Tip and StpC would act in synergy.

In addition to Lck, phosphorylated signal transducer and activator of transcription (STAT) factors 1 and 3 were immunoprecipitated with Lck and Tip-C484 (Lund *et al.*, 1997b). The implication of Y114 with constitutive active STATs, especially STAT3, and the role of STATs in growth regulation and oncogenesis in multiple cell types (Bowman *et al.*, 2000; Bromberg *et al.*, 2000) suggested a central role for Tip-induced STAT activity in viral T-cell transformation. However, recombinant herpesvirus saimiri C488 expressing Tip with a tyrosine-to-phenylalanine mutation at position 114 was able to transform primary human T-lymphocytes in the absence of STAT1 or STAT3 activation (Heck *et al.*, 2005). Thus, the essential function of Tip in lymphocyte transformation does not rely on Lck-mediated STAT1 or 3 phosphorylation. When Tip was highly expressed in stable *tip*-transfected Jurkat T-cells, low basal levels of tyrosine phosphorylation, impaired response to T-cell-receptor activation, and downregulation of CD3 and CD4 were observed. Tip further partially reversed the transformed phenotype of fibroblasts, which had lost contact inhibition after transfection with a constitutively active mutant of Lck. These effects were even more pronounced when Tip Tyrosine 114 was mutated in position 114 to Serine (Y114S) to enhance its binding to Lck (Guo *et al.*, 1997; Jung *et al.*, 1995b). Enhanced binding and activation of Lck by Tip carrying a Y114S might provide an explanation for the increased transformation efficiency, however, there were no differences in Lck binding for Tip Y114F (Heck *et al.*, 2006) and a Tip Y114 phosphopeptide exhibits no significant affinity for the SH2 domain of Lck (Bauer *et al.*, 2004).

The activation of Lck and the inhibition of T-cell signaling by Tip may be two different aspects of the same function, since the activation of Lck by Tip might trigger negative feedback mechanisms in stably transfected Jurkat cells expressing high levels of Tip, such as apoptosis (Hasham and Tsygankow, 2004). Another possible reason for this are the low expression levels of Tip in such HVS-transformed primary human and simian T-cells, while $p56^{lck}$ is abundantly expressed; any possible changes in the Lck enzymatic activity after binding of Tip may be masked by the excess free Lck. Tip function has evolved in the context of the natural infection of squirrel monkeys, where uncontrolled T-cell transformation seems not to occur. A better understanding of HVS biology in squirrel monkeys could help to elucidate this problem (Greve *et al.*, 2001).

A cellular Tip-associated protein of 65 kDa termed Tap could contribute to T-cell activation by Tip (Yoon *et al.*, 1997), although Tap is an RNA export factor that has no known T-cell-specific functions as yet (Grüter *et al.*, 1998). Coexpression of Tip and a recently identified lysosomal WD-repeat protein p80 at high levels in 293 and Jurkat cells induced enlarged endosomal vesicles and recruited Lck and TCR complex into these vesicles for trafficking or degradation. Since Tip is constitutively present in lipid rafts, it was also found that Tip can recruit p80 into lipid rafts; the C-terminus of Tip seems to interact with Lck to recruit TCR complex to lipid rafts, and TCR and Lck then may interact with p80 to initiate the aggregation and internalization of the lipid raft domain and thereby downregulate the TCR complex (Park *et al.*, 2002, 2003). The lipid raft association of Tip is essential for the TCR and CD4 downregulation but not for the inhibition of TCR signal transduction and the activation of STAT3 transcription factor (Cho *et al.*, 2006).

Growth transformation of human T-cells

Rapidly proliferating T-lymphoblastic tumor cell lines such as Jurkat (Schneider *et al.*, 1977) are frequently used as a cellular and biochemical model for primary human T-cells, although they display a strongly altered phenotype with respect to signal transduction, gene regulation and proliferation control. On the other hand, primary human T-cell culture is laborious and requires repeated stimulation with a mitogen or specific antigen in the presence of accessory cells expressing the appropriate MHC restriction elements. Since they are limited in their natural life span, it is rarely possible to grow primary T-lymphocytes to large numbers. A practical method of T-cell transformation became available through the observation that HVS strain C488 stimulated human T-lymphocytes to stable antigen-independent growth in culture (Biesinger *et al.*, 1992). These growth-transformed human T-cells retained many essential T-cell functions including the MHC-restricted antigen- specific reactivity of their parental T-cell clones (Bröker *et al.*, 1993; De Carli *et al.*, 1993; Weber *et al.*, 1993), they are not tumorigenic in nude or SCID mice, but could induce xenogenic graft-versus-host disiease similar as primary human T-cells (Huppes *et al.*, 1994). These observations have opened up a novel research direction which links T-cell biology, signal transduction pathways and transforming viral functions. Although HVS-transformed Old and New World monkey T-lymphocytes produce infectious viral particles in many cases, it was not possible to isolate virus from transformed human T-cell cultures (Biesinger *et al.*, 1992). Although the formal proof that the virus can never be reactivated from transformed human T-lymphocytes is difficult, neither treatment with phorbol esters or nucleoside analogues, nor other drugs that can cause reactivation of other viruses such as EBV or KSHV, nor specific or non-specific stimulation of the T-cells could induce virion production (Fickenscher *et al.*, 1996). StpC and Tip are the only viral proteins which have been regularly demonstrated in HVS-transformed human T-cells. The non-coding viral U-RNA genes (HSUR, HVS URNA) were abundantly expressed in a similar way to the EBER RNAs of EBV, but deletion of all HSUR did not influence virus replication or T-cell transformation (Ensser *et al.*, 1999). While the viral gene *stpC/tip* was strongly and inducibly transcribed into a bicistronic message (Fickenscher *et al.*, 1996; Medveczky *et al.*, 1993), other viral transcription was rarely detected. The gene *ie14/vsag* was abundantly transcribed for a few hours only after of stimulation of transformed human T-cells with phorbol ester, and the IE gene *ie57*, the early gene *orf50*, and the viral thymidylate synthase gene were found transcribed at extremely low abundance or only after additional T-cell stimulation (Knappe *et al.*, 1997; Thurau *et al.*, 2000). These findings argue for a strong block of virus replication in C488-transformed human T-cells that is downstream of the expression of the regulatory genes *orf50* and *ie57*.

The transformation procedure and the specific properties of HVS-transformed T-cells have been comprehensively reviewed (Fickenscher and Fleckenstein, 2001; Ensser *et al.*, 2002; Fickenscher and Fleckenstein, 2002; Ensser and Fleckenstein, 2004). Briefly, the infection of peripheral blood mononuclear cells, cord blood cells, thymocytes, or established T-cell clones by HVS C488 results in T-cell lines that continuously grow without restimulation with antigen or mitogen and do not require the presence of feeder or antigen presenting cells. The morphology of such lines resembles the irregular shape of T blasts, they carry non-integrated viral episomes in high copy number, and have a normal karyotype (Troidl *et al.*, 1994). The HVS strain C488 is commonly used for the targeted transformation of human T cells; other subgroup C virus strains were able to transform human T-cells, but to a varying extent (Fickenscher *et al.*, 1997). The surface phenotype of the transformed T-lymphocytes resembles mature, activated T cells, that are CD4+CD8− or CD4−CD8+ T cells and carry ab- or gd-type T-cell receptors; transformation of established T-cell clones demonstrated that the phenotype and HLA-restriction of the parental T-cells is conserved. If transformed by the same virus strain, ab and gd-clones were similar with respect to viral persistence, virus gene expression, proliferation and Th1-type cytokine production. The phenotype of HVS-transformed T-cells is remarkably stable for many months in culture, a significant technical advance over immortalization of human T-cells by the hybridoma technique or infection with HTLV-1 (Biesinger *et al.*, 1992; reviewed in Fickenscher and Fleckenstein, 2002). While many normal T-cell functions are preserved, a few specific cellular and biochemical features are changed in comparison with their parental cells. This relates to the hyper-responsiveness to CD2 ligation (Meuer *et al.*, 1984; Mittrücker *et al.*, 1992). Second, the protein tyrosine kinase $p53/56^{lyn}$ is expressed and enzymatically active in HVS transformed T-cells (Fickenscher *et al.*, 1997; Wiese *et al.*, 1996). Third, HVS-transformation shifts the range of cytokines secreted by stimulated T-cells towards a Th1 profile: IL-4 and IL-5 production is diminished and secretion of IL-2 and IFN-g is increased in comparison with parental cells. (Bröker *et al.*, 1993; De Carli *et al.*, 1993; Weber *et al.*, 1993). A novel IL-10-like cellular gene is specifically overexpressed in HVS-transformed T-cells, termed *ak155* or IL-26 (Knappe *et al.*,

2000b). Whereas IL-26 receptors have not been found on T-cells, this cytokine induces STAT activation in epithelial cells (Hör *et al.*, 2004). Thus, it is unlikely that IL-26 contributes to HVS-mediated T cell-transformation. However, this cytokine may be involved in the lymphocyte-epithelium interaction which is typical for various gamma herpesviruses.

T-cell transformation by HVS C488 was used to study T cells from primary human immunodeficiencies, and in many cases has been the only way to cultivate and amplify the patients' cells for further research. HVS transformed T-cell lines were established from patients with a variety of genetic T-cell defects (reviewed in Fickenscher and Fleckenstein 2001). HVS-transformed human CD4+ T-cells provide a productive system for T-lymphotropic viruses such as HHV-6 (F. Neipel and B. Fleckenstein, unpublished data) and human immunodeficiency virus (HIV) (Nick *et al.*, 1993).

Vectors for gene therapy

Both oncogenic and non-transforming HVS variants have been used as eukaryotic expression vectors. HVS vectors were used to define the transforming functions of the HTLV-1 X region and the *stpC* oncogene was successfully substituted by cellular *ras* (Guo *et al.*, 1998), by the K1 gene of KSHV/HHV-8 (Lee *et al.*, 1998), or by R1 of rhesus monkey rhadinovirus (RRV) (Fig. 60.2) (Damania *et al.*, 1999), in deletion mutants which still contained the *tip* gene. HVS has been used as a vector for growth hormone, for secreted alkaline phosphatase, anti-inflammatory cytokines and for green fluorescent protein (Desrosiers *et al.*, 1985b, 1996; Hoggard *et al.*, 2004; Stevenson *et al.*, 1999; Wieser *et al.*, 2005). A non-transforming replication-competent HVS vector expressing the bovine growth hormone in persistently infected simian T cells produced high amounts of the circulating bovine hormone in experimentally infected New World primates (Desrosiers *et al.*, 1985b, 1986). This suggested that episomally persisting, non-integrating HVS vectors could be used for therapy of hereditary genetic disorders, like cytokine receptor deficiencies (Altare *et al.*, 2001). HVS-vectors efficiently transduce human mesenchymal cells including bone marrow stromal cells (Frolova-Jones *et al.*, 2000) or haematopoietic precusors (Doody *et al.*, 2004); infection of human hematopoietic progenitor cells is somewhat less efficient and with a tendency towards partially differentiated cells (Stevenson *et al.*, 1999). Limited replication of HVS replication was observed in certain human cell types (Daniel *et al.*, 1975; Simmer *et al.*, 1991; Stevenson *et al.*, 1999). Although totipotent mouse embryonic stem (ES) cells were infected under drug selection with rather stable transgene expression (Stevenson *et al.*, 2000a), the infection of differentiated murine or rat cells is mostly inefficient.

The behavior of HVS C488 in various Old World monkey systems is of interest although macaque T-cell lines were shown to shed low amounts of virus particles in many cases (Alexander *et al.*, 1997; Knappe *et al.*, 2000a), reinfusion of autologous transformed T cells into the donor macaques did not induce disease, the infused T-cells persisted for extended periods, and the animals were protected against tumor induction by challenge with the HVS C488 virus (Knappe *et al.*, 2000a). Conversely an attempt to improve the biological safety of HVS vectors by inserting the prodrug activating thymidine kinase gene of herpes simplex virus looked promising in vitro (Hiller *et al.*, 2000b), but failed in vivo: the recombinant HVS-TK viruses not only showed no response to the administration of ganciclovir but induced tumors even more rapidly than the wild-type HVS control (Hiller *et al.*, 2000a). Nevertheless, since gene transfer into primary human T-cells by transfection or retroviral transduction methods remains difficult, the maintained functional phenotype of HVS-transformed T lymphocytes supports the use of HVS-vectors for human T-cells (reviewed in Fickenscher and Fleckenstein, 2002). Transformation-competent HVS-vectors might be valuable for the targeted amplification of functional human T-cells, or even for therapeutic redirection of human T-cell antigen specificity, as a tool for experimental cancer therapy applications.

Herpesvirus ateles

Natural occurrence and pathology

Herpesvirus ateles can be isolated at a high rate from spider monkeys (*Ateles (A.) spp.*) (for review see Fleckenstein and Desrosiers, 1982). Isolate #810 from *A. geoffroyii* (Melendez *et al.*, 1972) is officially classified as ateline herpesvirus type 2, whereas isolate #73 and related strains (#87, 93, 94) from *A. paniscus* were designated as ateline herpesvirus type 3 (Falk *et al.*, 1974). HVA replicates in OMK cells (Daniel *et al.*, 1976), but remains mostly cell-associated with syncytia formation. As a result, supernatants of such cultures have lower and unstable HVA titers. Like HVS, HVA is not pathogenic in its natural host, but causes acute T-cell lymphoma in various New World primate species including tamarins (*Saguinus oedipus*) and owl monkeys (*Aotus trivirgatus*) (Hunt *et al.*, 1972). The pathological changes are similar to those observed after HVS infection. In addition, HVA transforms T-cells of certain New World monkey species such as cotton-top tamarins in culture,

Table 60.1. Gammherpesviruses

Designation, Abbreviation	Host	Associated pathogenicity
Rhadinovirus species		
Kaposi's sarcoma associated herpesvirus, KSHV	Human	Kaposi's sarcoma, multicentric Castleman's disease, primary effusion lymphoma
Retroperitoneal fibromatosis-associated herpesvirus, RFHV, MnRhRV	Rhesus monkey	Retroperitoneal fibromatosis ?
Rhesus monkey rhadinovirus, RRV	Rhesus monkey	B-cell hyperplasia ?
Herpesvirus saimiri, HVS[a].	Squirrel monkey	T-cell lymphoma in other neotropical monkeys
Herpesvirus ateles, HVA	Spider monkey	T-cell lymphoma in other neotropical monkeys
Alcelaphine herpesvirus 1, AHV-1	Wildebeest	Malignant catarrhal fever in cattle
Alcelaphine herpesvirus 2, AHV-2	Hartebeest, Topi	
Ovine herpesvirus 2, OHV-2	Sheep	Malignant catarrhal fever in cattle and deer
Caprine herpesvirus 2	Goat	Chronic disease in deer ?
Bovine herpesvirus 4, BHV-4	Cattle	None reported
Equine herpesvirus 2 and 5, EHV-2, -5	Horse	Mononucleosis in horses?
Porcine lymphotropic herpesviruses, PLHV-1/2	Pig	None reported
Herpesvirus sylvilagus	Cottontail rabbit	Mononucleosis in rabbits ?
Murine gammaherpesvirus 68, MHV-68	Wood mouse, Bank vole	Mononucleosis in mice
Lymphocryptovirus Species		
Epstein–Barr Virus, EBV, HHV-4[a]	Human	B-cell lymphoma; NPC; Hodgkin's disease, other
Chimpanzee LCV, Herpesvirus pan	Chimpanzee	
Baboon LCV, Herpesvirus papio	Baboon	Spontaneous B-cell lymphoma (and in immunosuppressed animals)
Rhesus LCV, Cercopethine HV 15	Rhesus Monkey, Macaca mulatta	Spontaneous B-cell lymphoma (and in immunosuppressed animals)
cynomolgus LCVs (HVMF1, Cyno-EBV, Si-IIA-EBV)	Cynomolgus sp, Macaca fascicularis	Spontaneous B-cell lymphoma (and in immunosuppressed animals)
Marmoset LCV, CalHV3	Common marmoset	Spontaneous B-cell lymphoma
SmiLHV1	Gold-handed tamarin	Unknown
SscLHV1	Squirrel monkey	Unknown
PpiLHV1	White-faced saki	Unknown

[a]Type species

yielding cytotoxic T-cell lines (Falk *et al.*, 1978; reviewed in Fleckenstein and Desrosiers, 1982). Human T-cells have not been susceptible to transformation with various HVA strains.

Genome structure and replication

HVA strain #73 has a similar genome structure as HVS (Albrecht, 2000; Fleckenstein *et al.*, 1978a)(Fig. 60.1). The long unique L-DNA containing all the virus genes has 108 409 bp with 36.6% G+C, and the terminal repetitive H-DNA without coding capacity contains multiple tandem repeats of 1582 bp with 77.1% G+C. The HVA genome contains 73 ORFs and only two genes for U-RNA like transcripts (Albrecht, 2000; Albrecht *et al.*, 1999). While the viral SAG, Cyclin, GPCR homologues of HVS are conserved in HVA, it does not encode homologues to the ORF12, vIL17, vCD59 nor vFLIP (Fig. 60.1). The impression is rather convincing that HVA resembles an ancient variant of HVS which has either collected a smaller set of cell-homologous genes or has secondarily lost several genes.

Oncogenesis

In HVA strain #73, a spliced gene with two exons was detected in the left-terminal L- to H-DNA transition region. The derived viral protein shares local sequence homology with StpC and Tip of HVS and was therefore termed Tio ("two in one," Fig. 60.2). Tio is expressed in HVA-transformed simian T cells. After cotransfection, Tio

bound to and was phosphorylated by the Src kinases Lck or Src (Albrecht *et al.*, 1999). Human T-cells can be successfully transformed by recombinant HVS C488 in which the StpC and Tip genes were replaced by either the HVA #73 region containing the promoter and both exons of Tio, or a cDNA of Tio transcribed from a heterologous promoter; thus, Tio can substitute for both StpC and Tip in human T-cell transformation (Albrecht *et al.*, 2004). Tyrosine phosphorylation of Tio is required for transformation of human T-cells in the context of a recombinant HVS genome (Albrecht *et al.*, 2005), and it has been found that Tio induces NFkB signaling through direct interaction with TRAF6 (Heinemann *et al.*, 2006).

Lymphocryptoviruses of New World primates

Gammaherpesviruses that are closely related to EBV were recognized in several species of Old World primates from the mid-70s. Until recently the paradigm was that the lymphocryptoviruses (LCV) are restricted to Old World primates including humans (Wang *et al.*, 2001). However, a virus was isolated from common marmosets, both from healthy animals and animals with spontaneous B-cell lymphomas (Ramer *et al.*, 2000; Cho *et al.*, 2001); Related LCV have also been detected in several other New World primate species (de Thoisy *et al.*, 2003; Table 60.1). This marmoset LCV (CalHV3) clearly had a EBV-like genome structure, yet several Old World primate LCV specific genes (Rivailler *et al.*, 2002b) were absent in this isolate (Rivailler *et al.*, 2002a). Namely, strong divergences were found in the LMP1 and 2, and the EBNA-LP, -2, and -3, and the marmoset LCV does not have homologues to BCRF1/vIL10, BARF1/CSF-1R, BARF0, EBERs, and several other EBV genes of unknown function. Also, the organization of the putative marmoset LCV OriP-region is clearly distinct from the Old World primate LCV; the *family of repeats* are a succession of two different and diverged repeat subunits, and it lacks the *dyad symmetry* elements (Rivailler *et al.*, 2002a). In-vitro transformation of B-cells by human and simian OWP-LCV seems to be mostly restricted to the natural host or closely related species (Moghaddam *et al.*, 1998). However, experimental T-cell tumors can be induced in rabbits following infection by Cynomolgus (Cyno-EBV) and Baboon (HVP) -LCV (Hayashi *et al.*, 2001).

Conclusions

The γ-herpesvirus subgroups *Lymphocryptovirus* and *Rhadinovirus* occur in New World as well as Old World primates including humans. Several members of both virus subgroups are closely related to viral oncogenesis, though the simian viruses do not generally provide an uncomplicated model for multifaceted human diseases. Kaposi's sarcoma is a complex entity that may be caused by the direct transforming action of viral oncogenes as well as a chronic inflammatory reaction, which may be amended or modulated by KSHV-encoded or -induced cytokines and/or angiogenic factors. No comparable animal model could be based on the use of the KSHV-related primate γ2-herpesviruses. Although a comparable virus-associated acute peripheral pleomorphic T-cell lymphoma is not known yet in humans, Herpesvirus saimiri can serve as an experimental model for general tumor development in humans. The ability of certain HVS-strains to transform human T lymphocytes to provides a promising tool for laboratory studies in T-cell immunology, including inherited and acquired immunodeficiency. Stp, Tip and Tio represent new classes of membrane-bound viral oncoproteins, most likely as small adaptor polypeptides that proficiently dominate T-cell signaling. Recombinant rhadinoviruses can deliver foreign genes into primary human mesenchymal cells and T-lymphocytes; this may prepare the ground for future therapeutic applications of persisting rhadinoviral vectors in adoptive immunotherapy.

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EBV and KSHV – related herpesviruses in non-human primates

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Introduction

Herpesviruses can be found in primates throughout the animal kingdom.In the animal kingdom, the order of primates is classified into two suborders, the Prosimians and the Anthropoids (Fig. 61.1(a)). Prosimians are the earliest and most primitive of primates and are comprised of lemurs, lorises and tarsiers. Tarsiers share characteristics that are intermediate between the prosimians and the anthropoids, and hence are sometimes considered a third suborder. The Anthropoids are classified into platyrrhines (flat nosed) and catarrhines (downward pointing nose).

The platyrrhines are New World monkeys found exclusively in Mexico and Central and South America. This group includes tamarins, common marmosets, squirrel monkeys and spider monkeys. Evolution of the platyrrhines has been a subject of intense debate. Most believe that the origin and early diversification of platyrrhines occurred on the African continent. It is thought that the platyrrhines then crossed the Atlantic Ocean to the Americas at a time when sea levels were lower and the ocean ridges in the Atlantic were likely exposed as islands, creating pathways that were conducive to platyrrhine migration.

The catarrhines are sub-divided into Cercopithecoids or Old World monkeys (with tails) and Hominids (no tails) (Fig. 61.1(b)). Old World monkeys are found in both Africa and Asia. The rhesus monkey (*Macaca mulatta*) and the cynomolgus monkey (*Macaca fascicularis*) are examples of Old World primates found in Asia, while African green monkeys and baboons are Old World primates found exclusively in Africa. The Hominids include apes like chimpanzees, gibbons, gorillas, orangutans, and humans. Hominids are divided into three groups: the *pongidae* (orangutans) and the *hylabatidae* (gibbons) that live in Asia, and the *panidae,* including gorillas and chimpanzees, that live in Africa. The divergence of the platyrrhines and the catarrhines is thought to have occurred 35 million years ago and the divergence of the cercopithecoids from the hominoids is thought to have occurred about 25 million years ago. These estimates are based on paleontological fossil records, as well as sequence comparisons of mitochondrial genes (Schrago and Russo, 2003; Stewart and Disotell, 1998).

Nomenclature

A wide body of literature has identified the presence of the gammaherpesvirinae throughout the animal kingdom (Fig. 61.1). These viruses include members of both the lymphocryptovirus (LCV) and rhadinovirus sub-families of the gammaherpesvirinae. The name Lymphocrypto is derived from the Latin word *lympha* meaning "water" and the Greek word *kryptos* meaning "concealed," and the name Rhadino is derived from the Greek adjective *rhadinos*, meaning slender (ICTV website). Many of these non-human primate gammaherpesviruses are distinctly related to the human lymphocryptovirus, Epstein–Barr virus (EBV/HHV-4) and the human rhadinovirus, Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8).

The nomenclature used for the primate herpesviruses can be quite obscure. Names such as Herpesvirus papio and Herpesvirus pan are not specific since they do not clearly designate which herpesvirus is being identified. To clarify this, the International Committee on the Taxonomy of Viruses (ICTV) has suggested a defined nomenclature system which uses the family or sub-family of the natural host followed by the sequential number in order of discovery (ICTV website). Examples of this nomenclature system are *Cercopithecine herpesvirus* 17 which refers to the 17th herpesvirus found in rhesus macaques (also known as rhesus monkey rhadinovirus (RRV)), while *Cercopithecine herpesvirus* 15 refers to the 15th herpesvirus found in rhesus

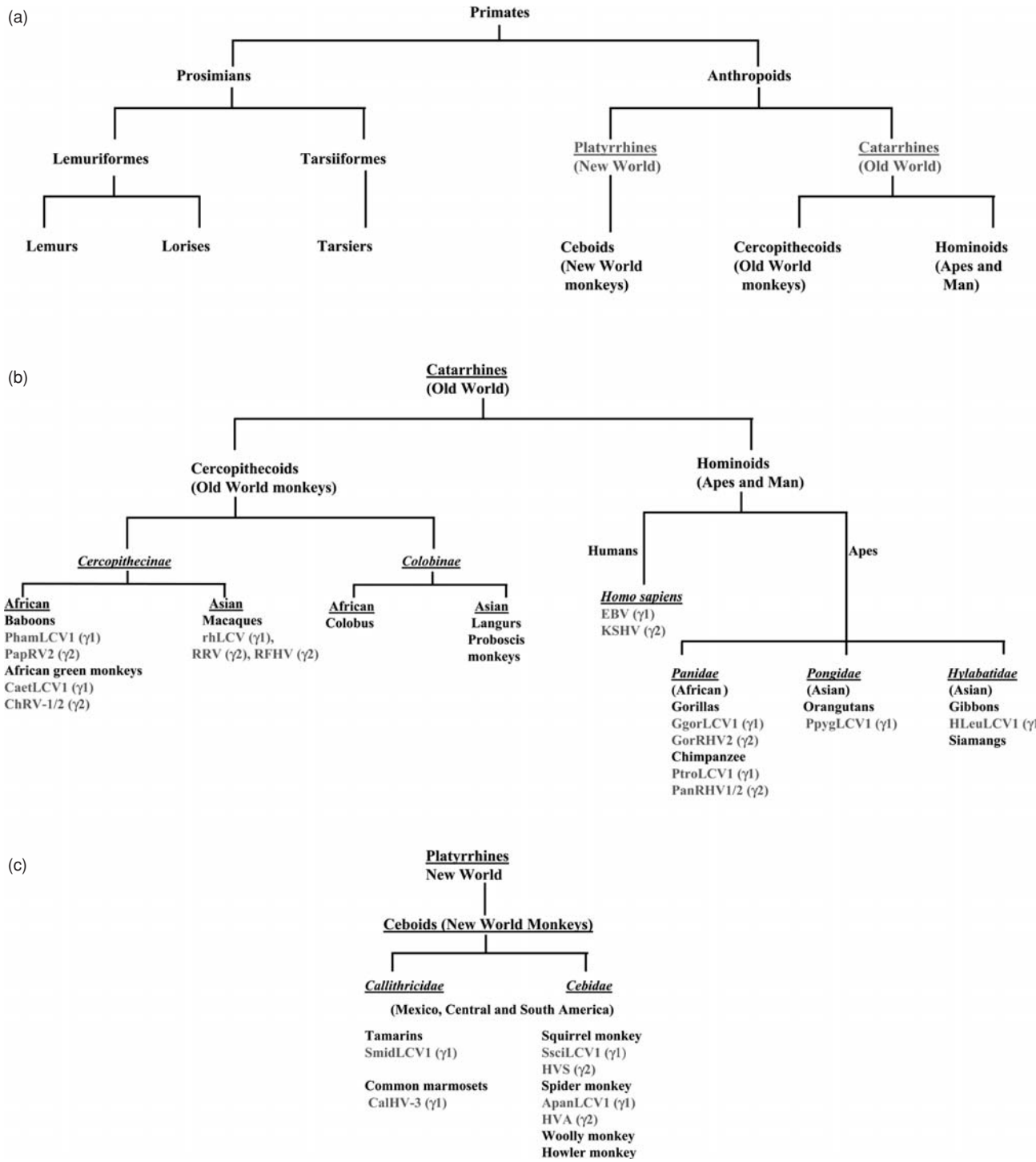

Fig. 61.1. Classification of primates and gammaherpesviruses in the Animal Kingdom. (a) Primates are classified into two groups; the Prosimians and the Anthropoids. (b) Classification of the Catarrhines or Old World Primates. The gammaherpesviruses found in the different primate species are shown in gray. (c) Classification of the Platyrrhines or New World Primates. The gammaherpesviruses found in the different primate species are shown in gray.

macaques also referred to as rhesus EBV. In order to facilitate recognition and easy usage, several investigators have combined the host species and virus for vernacular usage, e.g., baboon LCV. Further, due to the identification of multiple isolates of primate rhadinoviruses, some investigators have further chosen to numerically identify the lineage. For example, *Papio anubis* RV2 or PapRV2 is the alternate name for one of two rhadinoviruses found in baboons, whereas ChRV1 is the name of one of two rhadinoviruses found in African Green monkeys (Whitby *et al.*, 2003; Greensill *et al.*, 2000b) (Table 61.1).

Evolution of New and Old World Lymphocryptoviruses

The platyrrhines diverged from the catarrhines around 35 million years ago, and hence parallel the divergence of the New World and Old World lymphocryptoviruses. Thus, the rhesus LCV and human EBV genomes are more closely related to each other than the common marmoset LCV genome. However, it is clearly evident that both these viruses evolved from a common ancestral viral genome since many genes are conserved between these different lymphocryptoviruses. For example, several essential genes like EBV nuclear antigen 1 (EBNA-1), which is required for episome maintenance, and BZLF1 and BRLF1, which are required for viral replication, are conserved between the Old and New World lymphocryptoviruses and speak to the importance of their functions in the viral lifecycle (Tables 61.2 and 61.3). Other genes like the colony-stimulating factor 1 receptor (CF1R) homologue and the interleukin 10 (IL-10) homologue are present in only the Old World lymphocryptoviruses like EBV and rhesus LCV, but are absent from the New World lymphocryptovirus genome (Tables 61.2 and 61.3). This suggests that these two genes were captured by rhesus LCV or an ancestral virus after the divergence of the New and Old World LCV, and were selected for and retained by the human virus because they provide a biological advantage during the viral lifecycle.

Lymphocryptoviruses of Old World Monkeys

There are several lymphocryptoviruses that have been identified in both Old and New World monkeys (Table 61.1). Similar to their human counterpart, EBV, all these viruses are capable of immortalizing B cells in vitro. These viruses manifest both persistent infection and B-cell lymphomagenesis raising the speculation that a biological selection for these two characteristic properties of lymphocryptoviruses has existed throughout evolution. The first clue that Old World primates were indeed infected with EBV-related viruses came from studies demonstrating that the serum of several Old World primates exhibited antibody cross-reactivity against human EBV (Dunkel *et al.*, 1972; Kalter *et al.*, 1972; Naito *et al.*, 1971; Levy *et al.*, 1971; Landon and Malan, 1971). Lymphocryptoviruses were identified in baboons, common marmosets, gorillas and orangutans, and several LCV cell lines containing these viruses have been established in vitro (Rabin *et al.*, 1977a,b; Neubauer *et al.*, 1979; Rasheed *et al.*, 1977). Interestingly, viruses from these cell lines were shown to immortalize both autologous B cells, as well as B cells from closely related species (Falk *et al.*, 1977; Ishida and Yamamoto, 1987; Neubauer *et al.*, 1979; Rabin *et al.*, 1977a,b; Rangan *et al.*, 1986). Further, analysis of the restriction digestion patterns of the lymphocryptovirus herpesvirus papio (HVPapio) from baboons revealed that the viral genome was organized in a collinear fashion with human EBV and that the genomes shared 40% homology (Heller *et al.*, 1981; Heller and Kieff, 1981). To date, multiple lymphocryptoviruses have been identified from different Old World primates such as chimpanzees (Landon *et al.*, 1968), baboons (Vasiljeva *et al.*, 1974), African green monkeys (Bocker *et al.*, 1980), orangutans (Rasheed *et al.*, 1977) and gorillas (Neubauer *et al.*, 1979).

Other studies give credence to the close similarity between the simian and human lymphocryptoviruses. For example, rhesus macaques contain two rhesus lymphocryptoviruses (Type 1 and Type 2) similar to the situation in humans (Cho *et al.*, 1999). In the human population, there are two types of EBV isolates, EBV-1 and EBV-2 that can be distinguished by genetic polymorphisms in the EBNA genes (Dambaugh *et al.*, 1980, 1984, 1986; Zimber *et al.*, 1986; Rowe *et al.*,1989; Rowe and Clarke, 1989; Sample *et al.*, 1990). EBV-1 is more efficient in B cell immortalization than EBV-2 (Rickinson *et al.*, 1987; Cohen *et al.*, 1989). The two types of rhesus LCVs have similar genetic polymorphisms as seen in the human isolates, and share the same biological properties (Cho *et al.*, 1999). Thus, both the human and non-human primate lymphocryptoviruses share similar genomic organizations and biological properties, which suggests that they arose from the same ancestral lymphocryptovirus and that there exists a similar selection pressure for the evolution of two different LCVs in both humans and macaques.

The rhesus LCV genome

The complete rhesus LCV (type 1) genome has been cloned and sequenced (Rivailler *et al.*, 2002b). Rhesus LCV encodes

Table 61.1. Nomenclature of primate gammaherpesviruses

Species	Full Virus Name	Other Name	ICTV	Abbreviation	Reference
Old World Primates		**Lymphocryptoviruses**			
Chimpanzee	*Pan troglody*tes LCV1	Herpesvirus pan	*Pongine herpesvirus 1*	PtroLCV1	Greensill *et al.*, 2000a,b
Bonobo	*Pan paniscus* LCV1			PpanLCV1	Ehlers *et al.*, 2003
Gorilla	*Gorilla gorilla* LCV1	Gorilla herpesvirus	*Pongine herpesvirus 3*	GgorLCV1	Ehlers *et al.*, 2003
Gorilla	*Gorilla gorilla* LCV2	Gorilla herpesvirus	*Pongine herpesvirus 3*	GgorLCV2	Ehlers *et al.*, 2003
Orangutan	*Pongo pygmaeus* LCV1	Orangutan herpesvirus	*Pongine herpesvirus 2*	PpygLCV1	Ehlers *et al.*, 2003
White-cheeked gibbon	*Hylobates leucogenys* LCV1			HleuLCV1	Ehlers *et al.*, 2003
White-handed gibbon	*Hylobates lar* LCV1			HlarLCV1	Ehlers *et al.*, 2003
Hanuman langur	*Semnopithecus entellus* LCV1			SentLCV1	Ehlers *et al.*, 2003
Hamadryas baboon	*Papio hamadryas* LCV1	Herpesvirus papio	*Cercopithecine herpesvirus 12*	PhamLCV1	Ehlers *et al.*, 2003
Hamadryas baboon	*Papio hamadryas* LCV2	Herpesvirus papio	*Cercopithecine herpesvirus 12*	PhamLCV2	Ehlers *et al.*, 2003
Mandrill	*Mandrillus sphinx* LCV1			MsphLCV1	Ehlers *et al.*, 2003
Mandrill	*Mandrillus sphinx* LCV2			MsphLCV2	Ehlers *et al.*, 2003
Black and White colobus	*Colobus guereza* LCV1			CgueLCV1	Ehlers *et al.*, 2003
Western red colobus	*Piliocolobus badius* LCV1			PbadLCV1	Ehlers *et al.*, 2003
Black mangabey	*Cercocebus aterrimus* LCV1			CateLCV1	Ehlers *et al.*, 2003
Rhesus macaque	*Macaca mulatta* LCV1	Rhesus EBV	*Cercopithecine herpesvirus 15*	MmuLCV1	Franken *et al.*, 1996
Cynomolgus macaque	*Macaca fascicularis* LCV1			MfasLCV1	Ehlers *et al.*, 2003
Japanese macaque	*Macaca fuscata* LCV1			MfusLCV1	Ehlers *et al.*, 2003
Japanese macaque	*Macaca fuscata* LCV2			MfusLCV2	Ehlers *et al.*, 2003
Wanderoo	*Macaca silenus* LCV1			MsilLCV1	Ehlers *et al.*, 2003
Magot	*Macaca sylvanus* LCV1			MsylLCV1	Ehlers *et al.*, 2003
Tibet macaque	*Macaca tibetana* LCV1			MtibLCV1	Ehlers *et al.*, 2003
Patas monkey	*Erythrocebus patas* LCV1			EpatLCV1	Ehlers *et al.*, 2003
African green monkey	*Chlorocebus aethiops* LCV	African green monkey EBV-like virus	*Cercopithecine herpesvirus 14*	CaetLCV1	Bocker *et al.*, 1980
New World Primates					
Common squirrel monkey	*Saimiri sciureus* LCV1			SsciLCV1	Ehlers *et al.*, 2003
Common squirrel monkey	*Saimiri sciureus* LCV2			SsciLCV2	Ehlers *et al.*, 2003
Saki	*Pithecia pithecia* LCV1			PpitLCV1	Ehlers *et al.*, 2003
White-fronted capuchin	*Cebus albifrons* LCV1			CalbLCV1	Ehlers *et al.*, 2003
Black spider monkey	*Ateles paniscus* LCV1			ApanLCV1	Ehlers *et al.*, 2003
Black-penciled marmoset	*Callithrix penicillata* LCV1			CpenLCV1	Ehlers *et al.*, 2003
Common marmoset	*Callithrix jacchus* LCV1	Marmoset LCV		CjacLCV1/ CalHV3	Cho *et al.*, 2001
Red-handed tamarin	*Saguinus midas* LCV1			SmidLCV1	Ehlers *et al.*, 2003
		Rhadinoviruses			
Old World Primates					
Chimpanzee	*Pan troglodytes* RV1			PanRHV1a/ PtRV1	Greensill *et al.*, 2000a,b
Chimpanzee	*Pan troglodytes* RV1			PanRHV1b	Greensill *et al.*, 2000a,b
Chimpanzee	*Pan troglodytes* RV2	Pan Rhadino-herpesvirus 2		PanRHV2	Greensill *et al.*, 2000a,b

(*cont.*)

Table 61.1. (*cont.*)

Species	Full Virus Name	Other Name	ICTV	Abbreviation	Reference
Gorilla	*Gorilla gorilla* RV1			GorRHV1	Lacoste *et al.*, 2000a,b
Baboon	*Papio anubis* RV2			PapRV2	Whitby *et al.*, 2003
Mandrill	*Mandrillus sphinx* RHV1			MndRHV1	Lacoste *et al.*, 2000a,b
Mandrill	*Mandrillus sphinx* RHV2			MndRHV2	Lacoste *et al.*, 2000a,b
Rhesus macaque	*Macaca mulatta* RV1	Retroperitoneal fibromatosis herpesvirus		MmuRV1/ RFHVMm	Rose *et al.*, 1997
Rhesus macaque	*Macaca mulatta* RV2	Rhesus monkey rhadinovirus	*Cercopithecine herpesvirus 17*	RRV/MmuRV2	Desrosiers *et al.*, 1997
Cynomolgus macaque	*Macaca fascicularis* RV2			MGVMf	Rose *et al.*, 2003
Pig-tailed macaque	Macaca nemestrina RV1			MneRV1/ RFHVMn	Rose *et al.*, 1997
Pig-tailed macaque	Macaca nemestrina RV2			MneRRV/ MneRV2	Schultz *et al.*, 2000
African green monkey	*Chlorocebus aethiops* RV1	Chlorocebus rhadinovirus 1		ChRV1	Greensill *et al.*, 2000a,b
African green monkey	*Chlorocebus aethiops* RV2	Chlorocebus rhadinovirus 2		ChRV2	Greensill *et al.*, 2000a,b
New World Primates					
Common squirrel monkey	*Saimiri sciureus* RV	Herpesvirus saimiri	*Saimiriine herpesvirus 2*	HVS/SaHV-2	Albrecht and Fleckenstein., 1990
Spider monkey	*Ateles paniscus* RV	Herpesvirus ateles 3	*Ateline herpesvirus 3*	HVA3	Albrecht and Fleckenstein., 2000

In order to achieve consistency, the full virus name indicates first the species in which the virus was found, and then the virus itself; RV = rhadinovirus and LCV = lymphocryptovirus. Lineages 1 and 2 are also indicated. The other name and abbreviation refers to the name and abbreviations assigned by different laboratories who found the same or similar virus.

Table 61.2. A comparison of proteins encoded by the rhesus and human lymphocryptoviruses

Rhesus LCV	% aa similarity to EBV	Function
LMP1	32.4	Transforming protein
LMP2A	57	Signal modulator
EBER-1	–	Small RNA
EBER-2	–	Small RNA
EBNA-1	46.3	Episomal maintenance
EBNA-2	29.8	Nuclear protein
EBNA-3A	29.4	Nuclear protein
EBNA-3B	30.5	Nuclear protein
EBNA-3C	31.2	Nuclear protein
BCRF1	84.1	IL-10 homologue
BARF1	75	CF1R homologue
BHRF1	72.8	Bcl-2 homologue
BALF1	84.1	Bcl-2 homologue
BZLF1	71.3	Transcription factor
BRLF1	76.3	Transcription factor
BALF5	94.8	DNA polymerase

eighty open reading frames (ORFs) and each ORF shares homology to a corresponding gene in human EBV. The average gene homology between EBV and rhesus LCV is 75.6% (Rivailler *et al.*, 2002b). Each ORF is located at an equivalent position in the viral genome (Rivailler *et al.*, 2002b). Many of the latent and lytic genes of rhesus LCV are conserved with those of human EBV. Latent genes include those of the EBV nuclear antigens, EBNA-1, 2, 3A, 3B, 3C, the EBV-encoded small RNAs (EBERs) and the latent membrane proteins LMP1, 2A and 2B (Rivailler *et al.*, 1999, 2002b; Blake *et al.*, 1999; Cho *et al.* 1999; Franken *et al.*, 1996; Jiang *et al.*, 2000; Peng *et al.*, 2000). Both the rhesus LCV and human EBV genomes have four homologues of cellular genes: CSF1R (BARF1), two bcl-2 homologues (BHRF1 and BALF1) and an IL-10 homologue (BCRF1) (Rivailler *et al.*, 2002b). Interestingly, although the bcl-2 homologues are conserved between the Old and New World lymphocryptoviruses (Tables 61.2 and 61.3), the IL-10 homologue and the colony-stimulating factor 1 receptor were recently acquired genes, and are not present in an LCV genome isolated from a

Table 61.3. A comparison of proteins encoded by the human and marmoset lymphocryptoviruses

Marmoset LCV	% aa similarity to EBV	Function	Positional homologue in EBV
C1	–	Unique gene (Transforming gene)	LMP-1
C2	–	Unique gene	BILF2
C3	–	Unique gene	EBNA-3
C4	–	Unique gene	BHLF1
C5	–	Unique gene	EBNA-2
C6	–	Unique gene	none
C7	–	Unique gene	LMP-2
ORF39 (EBNA-1)	36.0	Episomal maintenance	EBNA-1
ORF 64 (BHRF1)	20.5	Bcl-2 homologue	BHRF1
ORF 1 (BALF1)	27.6	Bcl-2 homologue	BALF1
ORF 43 (BZLF1)	29.0	Transcription factor	BZLF1
ORF 42 (BRLF1)	39.0	Transcription factor	BRLF1
ORF 5 (BALF5)	73.5	DNA polymerase	BALF5

common marmoset (Rivailler *et al.*, 2002b) (Tables 61.2 and 61.3). Table 61.2 shows a list of important genes encoded by rhesus LCV and their function.

Latency, immune-modulatory and transforming genes of rhesus LCV

Epstein–Barr virus nuclear antigen-1 (EBNA-1)

The latency gene, EBV EBNA-1 is a critical gene required for establishment and maintenance of the viral genome in the latent state. Both baboon LCV (*Cercopithecine herpesvirus 12* or herpesvirus papio) and rhesus LCV (*Cercopithecine herpesvirus 15*) encode homologues of EBV EBNA-1 that are highly conserved (Blake *et al.*, 1999; Yates *et al.*, 1996). The same is true for the EBNA-1 protein from a lymphocryptovirus infecting cynomolgus monkeys (Ohara *et al.*, 2000). Interestingly, these simian viral EBNA-1 proteins are slightly smaller than the human viral protein due to differences in the glycine-alanine (GAR) repeat domain. The baboon and rhesus LCV EBNA-1 proteins can both function to support EBV ori-P-dependent plasmid replication and maintenance, similar to EBV EBNA-1 (Marechal *et al.*, 1999; Ruf *et al.*, 1999). In addition, the molecular mechanisms governing latent gene expression such as the EBNA-1 and EBNA-2 transcripts are also conserved among the primate LCVs (Ruf *et al.*, 1999; Fuentes-Panana *et al.*, 1999).

However, one difference between these proteins is that, although the EBV EBNA-1 protein can inhibit antigen presentation to escape CTL surveillance (Levitskaya *et al.*, 1995), this immunomodulatory function is not conserved in the rhesus LCV EBNA-1 protein (Blake *et al.*, 1999) using in vitro assays. However, in naturally and experimentally infected rhesus macaques, the LCV EBNA-1 protein appeared to play a role in immune evasion in vivo (Fogg *et al.*, 2005).

Epstein–Barr virus nuclear antigen-2 (EBNA-2)

Despite the fact that the EBNA-2 protein in the rhesus and baboon LCVs show only limited homology to each other and to human EBV, their functional characteristics such as their interactions with the transcription factor RBP-Jk have been retained (Ling *et al.*, 1993; Ling and Hayward, 1995; Cho *et al.*, 1999; Peng *et al.*, 2000). The polyproline repeat in the rhesus and baboon EBNA-2 proteins is shorter than the EBV EBNA-2 protein (Peng *et al.*, 2000). However, since this region appears to be dispensable for transactivation and B cell immortalization (Yalamanchili *et al.*, 1994) the significance of this divergence may be minimal. As mentioned above, genetic polymorphisms in the EBNA-2 genes have suggested the presence of two types of LCVs in rhesus macaques, similar to the situation in humans.

Epstein–Barr virus nuclear antigen- 3A,3B,3C (EBNA-3A, 3B, 3C)

The rhesus LCV latency-associated genes, EBNA-3A, 3B and 3C, show loose homology to human EBV EBNA-3A, 3B and 3C genes. Again, despite the limited conservation in sequence among these proteins, the interaction with RBP-Jk has been retained (Dalbies-Tran *et al.*, 2001; Zhao *et al.*, 2003; Jiang *et al.*, 2000). Further, the transactivation function of EBNA-3C has also been conserved between the rhesus, baboon and human viral proteins, and all three

proteins can interact with the Spi proteins (Zhao *et al.*, 2003). Both non-human primate 3C proteins can support transcriptional activation mediated by the Spi proteins in the presence of EBNA-2 (Zhao *et al.*, 2003). Despite these similarities, gene replacement studies demonstrated that the rhesus LCV EBNA-3 genes were unable to functionally substitute for the EBV EBNA-3 genes in the EBV genome, for immortalization of human B-cells (Jiang *et al.*, 2000). However, the ability of this recombinant virus to immortalize rhesus B cells was not tested.

EBV latent membrane protein (LMP-1)

The EBV-encoded latent membrane protein 1 (LMP1) structurally and functionally resembles a constitutively active TNF family receptor. LMP1 aggregates in the plasma membrane and can activate a multitude of signaling pathways in the cell resulting in cell proliferation and transformation. Sequence analysis of the LMP-1 proteins from baboons (*Cercopithicine herpesvirus 12* or herpesvirus papio) and rhesus monkeys (*Cercopithicine herpesvirus 15*) showed that although the transmembrane domains of these proteins are conserved with that of EBV LMP-1, there is great divergence within the carboxy-terminal cytoplasmic domains of these proteins (Franken *et al.*, 1996). The C-terminal domain of EBV LMP1 has been shown to be essential for B-cell immortalization and interaction with members of the tumor necrosis factor receptor family. A comparative study of the simian LMP-1 proteins with EBV LMP-1 showed that the simian LCV LMP-1 proteins could induce NF-kB activity, bind tumor necrosis factor associated factor −3 (TRAF3) and induce ICAM1 expression (Franken *et al.*, 1996). This is likely through the multiple TRAF3 binding sites (PXQXT/S) that are contained within the simian LCV LMP1 C-terminal domain (Franken *et al.*, 1996). A similar study performed with an LCV LMP1 protein from cynomolgus monkeys (*Macaca fascicularis*) shows that this simian LMP1 protein also contains two PXQXT/S motifs and retains its ability to activate the NF-kB pathway (Faucher *et al.*, 2002). It has recently been reported that the transmembrane domain of EBV LMP1, specifically FWLY (amino acids 38–41), is critical for signal transduction which includes raft localization of the viral protein, TRAF3 interaction and NF-kB activation (Yasui *et al.*, 2004). Although the identical FWLY motif is not present in either the baboon or rhesus LCV LMP-1 proteins, the simian LMP-1 viral proteins may contain motifs that are functionally similar to FWLY, since they can activate NF-kB. Further, Eliopoulos and Young (1998) have reported that LMP1 homologues from the baboon and rhesus EBV are also capable of activating the c-Jun N-terminal kinase (JNK) pathway (Eliopoulos and Young, 1998). Thus, activation of the NF-kB and JNK pathways by the primate LMP1 proteins is an important function that has been conserved throughout evolution.

EBV latent membrane protein-2 (LMP-2)

The LMP2A homologues in the baboon and rhesus LCV genomes show homology to EBV LMP2 and contain an immunoreceptor tyrosine based activation motif (ITAM) (Franken *et al.*, 1996; Rivailler *et al.*, 1999). All three primate LCV LMP2A proteins contain 12 transmembrane domains, and although the amino acids in the cytoplasmic domain are quite different, the ITAM and proline-rich domains are well conserved. These conserved motifs/domains in EBV LMP2A have been shown to be required for interaction with protein tyrosine kinases (Portis *et al.*, 2002) and are essential for LMP2A function. Moreover, the rhesus LCV LMP2B gene is located at an equivalent genomic position as EBV LMP2B, and the EBNA-2 responsiveness and the bidirectional nature of the LMP1-LMP2B promoter is conserved between the rhesus and human lymphocryptoviruses (Rivailler *et al.*, 1999).

Rhesus LCV as an animal model system for EBV

Historically, several investigators attempted and failed to infect Old World primates such as baboons and rhesus macaques with human EBV (Frank *et al.*, 1976; Gerber *et al.*, 1969; Levine *et al.*, 1980). This failure to establish EBV infection in Old World primates may be due to the route of inoculation used in these studies, pre-existing antibody cross-reactivity due to natural LCV infection in the primates, or the utilization of non-transforming deletion mutants of EBV, namely P3HR1 (Levine *et al.*, 1980). It is also possible that there is a species restriction imposed on EBV infection of non-human primates, since LCVs tend to be competent for immortalization of B-cells from the same or closely related species, but immortalization potential varies for B-cells from a more divergent species. For example, Moghaddam *et al.* (1998) found that rhesus LCV did not immortalize human B cells, and human EBV could not immortalize rhesus monkey B cells (Moghaddam *et al.*, 1998). Further, since the rhesus LCV was able to infect human B cells, the species restriction was after virus penetration (Moghaddam *et al.*, 1998). Rhesus and baboon LCVs have been reported to be incapable of immortalizing human B-cells (Falk *et al.*, 1977), although other reports suggest that this is possible at a much lower frequency (Rabin *et al.*, 1977a; Gerber *et al.*, 1977). In a similar manner, EBV has been shown to immortalize B cells from chimpanzees, which are more closely

related to humans, and chimpanzee LCV can immortalize human B-cells (Gerber *et al.*, 1977; Ishida and Yamamoto, 1987). Likewise, baboon LCV can immortalize B-cells from gibbons, rhesus and cynomolgus macaques (Gerber *et al.*, 1977).

In light of the fact that human EBV fails to establish long-term infection in rhesus macaques, rhesus LCV serves as a valuable animal model to study EBV pathogenesis since successful experimental infection of naïve rhesus macaques with rhesus LCV has been previously reported (Moghaddam *et al.*, 1997). Naïve rhesus macaques were orally infected with rhesus LCV and this resulted in an acute and persistent LCV infection, which closely resembled that seen with primary EBV infection of humans. Initial viral load peaked between 4 to 21 days and declined to almost undetectable levels over a 3-month period (Rao *et al.*, 2000). Immune activation was evidenced three days after oral inoculation by the presence of T-cells expressing IL-2, IL-10 and gamma interferon. Activated CTL activity against LCV infected cells was observed in the first few weeks post-infection similar to what has been described for infectious mononucleosis patients in the human population (Wang, 2001). In addition, antibody responses against EBNA-2 and viral capsid antigens develop within 2 weeks post-inoculation (Moghaddam *et al.*, 1997; Mohle *et al.*, 1997; Rao *et al.*, 2000). Persistent viral infection could be detected in the oropharynx of infected animals by PCR (Cho *et al.*, 1999; Moghaddam *et al.*, 1997; Mohle *et al.*, 1997; Rao *et al.*, 2000) and in B-cells, and a percentage of these infected B cells were immortalized and could be grown in tissue culture (Moghaddam *et al.*, 1997; Mohle *et al.*, 1997). Persistent infection as measured by RT-PCR of the rhesus LCV EBER genes in peripheral blood mononuclear cells (PBMCs) was detected three years post-infection (Rao *et al.*, 2000). Thus, clinically speaking, primary, acute and persistent infection of macaques with rhesus LCV appears to closely mimic EBV infection of humans, further validating the use of rhesus LCV as an animal model system to study EBV pathogenesis.

The potential to use the rhesus LCV animal model system to study EBV-associated tumorigenesis is the subject of current and future investigation. Rivailler *et al.* reported that four immunosuppressed macaques infected orally with LCV failed to debelop lymphoproliferative disease. However, intravenous inoculation of autologous LCV immortalized B cells in four SHIV89.6P-infected animals resulted in one severely immunosuppressed animal developing an aggressive monoclonal LCV-positive lymphoma (Rivailler *et al.*, 2004). Feichtinger *et al.* (1992a,b) have shown that when cynomolgus monkeys naturally infected with LCV were infected with simian immunodeficiency virus (SIV), malignant B cell lymphomas containing DNA which cross-hybridized with human EBV were detected. In another study, squamous epithelial proliferative lesions in SIV-infected rhesus monkeys were also shown to contain EBV-like sequences by immunohistochemistry and *in situ* hybridization (Baskin *et al.*, 1995). A study on SIV-infected monkeys at the Tulane Primate Center showed that SIV-infected macaques have a higher rhesus LCV load in PBMCs than uninfected animals, but that the virus load varies widely among animals during disease progression (Habis *et al.*, 2000).

The current availability of the genomic sequence of rhesus LCV will facilitate the construction of recombinant LCVs that can be tested in rhesus macaques. Such studies will provide an understanding of the contribution of individual genes to the life cycle of these lymphocryptoviruses and serve to verify rhesus LCV as a tractable system to model EBV pathogenesis.

Other lymphocryptoviruses in Old World primates

A recent study by Ehlers *et al.* (2003) has identified the presence of novel lymphocryptoviruses in several species including baboons, chimpanzees, and gorillas (Table 61.1). Interestingly, this group found evidence for a second lymphocryptovirus in gorillas, baboons, mandrills and Japanese macaques (Fig. 61.2). Together these data raise the possibility that another yet to be discovered human lymphocryptovirus, different from the type 1 and 2 human EBV strains, may exist in the human population. This theme will be revisited when discussing the family of primate rhadinoviruses.

Lymphocryptoviruses of New World monkeys

Early serological studies had indicated that there was no evidence for the presence of lymphocryptoviruses in New World primates since there was no antibody cross-reactivity with human EBV from sera of New World monkeys (Frank *et al.*, 1976). However, Ramer *et al.* (2000) recently reported the identification of novel viral DNA sequences from common marmoset monkeys (*Callithrix jaccus)* afflicted with spontaneous B-cell lymphomas. These sequences were most closely related to EBV and suggested the presence of a novel lymphocryptovirus in New World primates. Subsequently, the first EBV-related herpesvirus in New World primates was cloned from common marmoset monkeys (Cho *et al.*, 2001; Jenson *et al.*, 2002). This virus was formally named Callitrichine herpesvirus

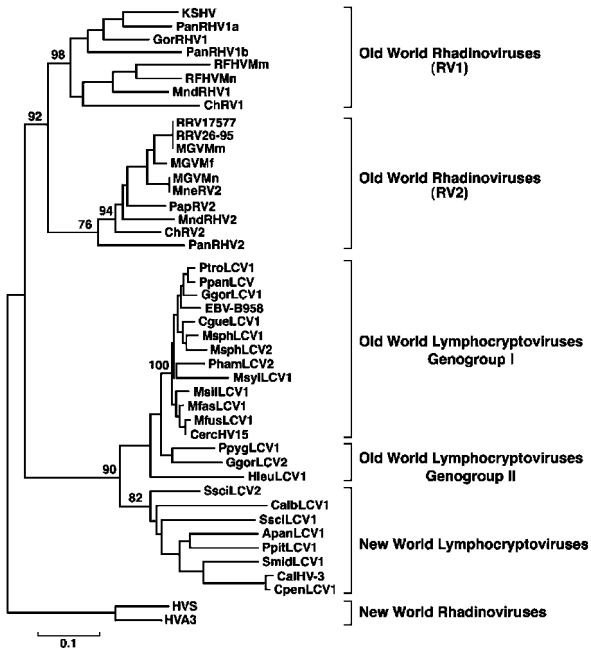

Fig. 61.2. Phylogenetic analysis of the gammaherpesviruses. Forty-four DNA polymerase nucleotide sequences (400bp) were aligned using the Clustal W matrix and edited in GeneDoc (v.2.6) to remove gaps. The tree was rooted using the New World Rhadinoviruses, HVS and HVA3 as the Outgroup. The Neighbor-Joining tree was constructed in Mega 2.1 from pairwise sequence distances calculated using the Kimura 2-parameter (K80) method. Bootstrap values were determined by 100 replica samplings. Sequences included in the phylogenetic analysis: KSHV (U75698); PanRHV1a (*Pan troglodytes*, AF250879); GorRHV1 (*Gorilla gorilla*, AF250886); PanRHV1b (*Pan troglodytes*, AF250882); RFHVMm (*Macaca mulatta*, AF005479); MndRHV1 (*Mandrillus sphinx*, AF282943); ChRV1 (*Chlorocebus aethiops*, AJ251573); RRV17577 (*Macaca mulatta*, AF083501); RRV26-95 (AF029302); MGVMm (*Macaca mulatta*, AF159033); MGVMf (*Macaca fascicularis*, AF159032); MGVMn (*Macaca nemestrina*, AF159031); MneRV2 (*Macaca nemestrina*, AF204167); PapRV2 (*Papio anubis*, AY270026); MndRHV2 (*Mandrillus sphinx*, AF282939); ChRV2 (*Chlorocebus aethiops*, AJ251574); PanRHV2 (*Pan troglodytes*, AF346490); PtroLCV1 (*Pan troglodytes*, AF534226); PpanLCV1 (*Pan paniscus*, AF534220); GgorLCV1 (*Gorilla gorilla*, AF534225); EBV-B95.8 (V01555); CgueLCV1 (*Colobus guereza*, AF534219); MsphLCV1 (*Mandrillus sphinx*, AF534227); MsphLCV2 (*Mandrillus sphinx*, AY174066); PhamLCV2 (*Papio hamadryas*, AF534229); MsylLCV1 (*Macaca sylvanus*, AY172956); MsilLCV1 (*Macaca silenus*, AF534222); MfasLCV1 (*Macaca fascicularis*, AF534221); MfusLCV1 (*Macaca fuscata*, AF534224); CercHV-15 (*Cercopithicine*, AY037858); PpygLCV1 (*Pongo pygamaeus*, AY129398); GgorLCV2 (*Gorilla gorilla*, AY129395); HleuLCV1 (*Hylobates leucogenys*, AY174068); SsciLCV2 (*Saimiri sciureus*, AY139024); CalbLCV1 (*Cebus albifrons*, AY139027); SsciLCV1 (*Saimiri sciureus*, AY172953); ApanLCV1 3 or CalHV3. A closely related virus to CalHV3 was also seen in squirrel monkeys (*Saimiri scireus*) (Cho *et al.*, 2001). Sequencing of the CalHV3 or marmoset LCV DNA revealed that the genomic organization was similar to that of EBV (Rivailler *et al.*, 2002a). Sequence analysis of the 73 open-reading frames (ORFs) revealed that although many genes showed high homology to genes in EBV, there were some striking differences between the two genomes as well.

The marmoset LCV (CalHV3) genome

The marmoset LCV genome is composed of approximately 160 000 nucleotides. Of the 73 ORFs found in the marmoset LCV genome, 59 of these share homology to genes found in all herpesviruses. Six additional genes encoded by ORFs 1,6,39,43,44 and 45 show homology to EBV BALF1, BILF1, EBNA-1, BZLF1, BZLF2 and gp350, respectively (Rivailler *et al.*, 2002a). The eight other marmoset ORFs show no sequence relatedness to either cellular or viral genes and were named C0 through C7 (Table 61.3). Based on their genomic position only, C0, C1,C2, C3,C4, C5 and C7 are in the equivalent genomic locations as EBV EBNA-LP, LMP1, BILF-2, EBNA-3, BHLF1, EBNA-2 and LMP2, respectively. In addition to encoding unique genes, there are also eleven EBV genes that are not present in marmoset LCV. These include the EBERs, BARF0, BCRF1, BARF1, and BDLF3 (Rivailler *et al.*, 2002a).

CalHV3 C1

C1 is a positional homologue of EBV LMP1. Although it shares no homology with LMP1 at the amino acid level and does not contain the PXQXT/S motifs that are contained in the C-terminus of the rhesus LCV and human EBV, C1 is a functional homologue of LMP1. It can transform rodent fibroblasts in vitro and can also induce NF-kB activity to similar levels as LMP1. Hence, C1 can interact with the TRAFs through an alternative TRAF binding motif (Wang *et al.*, 2001).

CalHV3 ORF39 (EBNA-1)

The CalHV3 EBNA-1 protein shows homology to EBV EBNA-1 in the C-terminal domain and the GR-rich

←

Fig. 61.2. (*cont.*) (*Ateles paniscus*, AY139028); PpitLCV1 (*Pithecia pithecia*, AY139025); SmidLCV1 (AY166693); CalHV-3 (*Cercopithicine*, AY049065); CpenLCV1 (*Callithrix penicillata*, AY139026); HVS (*Saimiri*, M31122); HVA3 (*Ateline*, AF083424). We thank R.K. Bagni and D. Whitby for help with the construction of the phylogenetic tree.

domains, which in EBV EBNA-1, are required for episomal maintenance (Wang *et al.*, 2001). However, the CalHV3 EBNA-1 homolog does not contain the Gly-Ala repeat region, which has previously been shown to be involved in immune-modulation (Levitskaya *et al.*, 1995).

CalHV3 C5

CalHV3 C5 is a positional homologue of EBNA-2 and shares no relatedness at the amino acid level. However like EBV EBNA-2, the C5 protein has a cluster of C-terminal acidic residues that may be important for transcriptional transactivation (Rivailler *et al.*, 2002a). Unlike the EBV and rhesus LCV EBNA-2, C5 is missing the polyproline repeat, which is present in EBV EBNA-2 (Yalamanchili *et al.*, 1996).

CalHV3 C7

C7 is a positional homologue of EBV LMP2. Similar to LMP2, it contains 12 transmembrane domains but a shorter N-terminus and a longer C-terminus (Rivailler *et al.*, 2002a). The latter contains five tyrosine residues, three of which may serve as part of Src-homology-2 (SH2) binding motifs (Rivailler *et al.*, 2002a).

New World primates as an animal model system for EBV

Historically, an extensive body of work has been published on using New World primates as an animal model system to study EBV (Shope *et al.*, 1973; Miller *et al.*, 1972, 1973). EBV readily infects and immortalizes B-cells from common marmosets (Desgranges *et al.*, 1976; Rabin *et al.*, 1977b). In fact one of the most widely used EBV-infected cell lines, B95-8, is a marmoset B-cell line infected with human EBV (Miller *et al.*, 1972). New World monkey species, including the cotton-top tamarin (*Sanguinus oedipus*), and owl monkey (*Aotus trivirgatus*) develop B-cell lymphomas upon infection with EBV (Cleary *et al.*, 1985; Johnson *et al.*, 1983; Epstein *et al.*, 1973a,b; Miller *et al.*, 1977; Werner *et al.*, 1975). Some publications suggest that common marmosets (*Callithrix jacchus*) also develop lymphoproliferative disease when infected with EBV DNA, and that lymphosarcomas in these animals contain EBV DNA (Falk *et al.*, 1976). Conversely, others have reported no substantial evidence of lymphoproliferative disease or lymphoma in EBV-infected common marmosets (de-The *et al.*, 1980; Ablashi *et al.*, 1978). Infection of common marmosets is thought to serve as a model for primary and persistent EBV infection (Wedderburn *et al.*, 1984).

Inconsistency in the induction of tumors by EBV in common marmosets, suggests that the cotton top tamarin is a more susceptible animal model for EBV oncogenesis. However, the cotton top tamarin was declared endangered in 1973 following the exportation of twenty to forty thousand tamarins from Colombia to the United States for use in biomedical research related to colonic adenocarcinoma (Hernandez-Camacho, 1976; Clapp *et al.*, 1982). Hence, their potential value as an animal model for EBV pathogenesis is limited due to their unavailability as well as the expense of the monkeys.

A comprehensive study on lymphocryptoviruses (LCV) that infect New and Old World primates has identified the presence of many lymphocryptoviruses from a multitude of primates (Ehlers *et al.*, 2003; de Thoisy *et al.*, 2003) (Table 61.1). These studies were done using degenerate PCR and revealed that some New and Old World monkeys are infected with two different lymphocryptoviruses, denoted as LCV1 and LCV2 (Table 61.1). In each primate species examined, one LCV virus was more closely related to human EBV and grouped in the same genogroup as EBV (Fig. 61.2), while the viruses that were less closely related to EBV all grouped together in a second lymphocryptovirus genogroup (Ehlers *et al.*, 2003) (Fig. 61.2).

Evolution of New and Old World rhadinoviruses

The Old World rhadinoviruses like RRV, RFHV and KSHV share more sequence relatedness amongst each other than with the New World rhadinovirus, HVS (see chapter on *Herpesvirus saimiri*), exemplifying the split of the platyrrhines and catarrhines 35 million years ago. Similar to the situation for the divergence of the lymphocryptoviruses, the rhadinoviral genomes of Old and New World primates both retain key viral genes such as Orf73 or Latency associated nuclear antigen (LANA), which is required for episomal maintenance of the viral genome, and Orf50/Rta which is required for viral replication. However, other proteins like viral interleukin 6 (vIL-6), macrophage inflammatory proteins (MIPs I, II and III), and the viral interferon regulatory factors (vIRFs) are only encoded by the Old World rhadinoviral genomes, RRV and KSHV, but are not present in the HVS genome, suggesting that these genes are more recent acquisitions in the rhadinovirus family, occurring after the split of the Old and New World primates. These proteins likely play key roles in the escape from immune surveillance and survival of the rhadinoviruses in their host species, and thus have been retained by KSHV, the most recent addition to the rhadinovirus evolutionary tree.

Rhadinoviruses of Old World primates

A wide body of literature supports the prevailing view that rhadinoviruses can be found throughout the animal kingdom in both Old and New World primates (Fig. 61.1). Rhadinoviruses that infect New World monkeys include herpesvirus saimiri (HVS) whose natural host is the squirrel monkey and herpesvirus ateles (HVA), which infects spider monkeys (Table 61.1) (Melendez *et al.*, 1969, 1972). Rhadinoviruses that infect Old World monkeys include rhesus monkey rhadinovirus (RRV) and retroperitoneal fibromatosis herpesvirus (RFHV) (Rose *et al.*, 1997; Desrosiers *et al.*, 1997).

Phylogenetic analysis

Among Old World primates, rhesus monkey rhadinovirus (RRV) and closely related viruses like RFHV, are gamma-2 herpesviruses of the macaque genus (Rose *et al.*, 1997; Desrosiers *et al.*, 1997). The similarity between the genomes of RRV and KSHV is high and all of the open reading frames (ORFs) in RRV have at least one homologue in KSHV (Alexander *et al.*, 2000; Searles *et al.*, 1999). RRV replicates lytically in cell culture and multiple isolates have been obtained independently by different laboratories (Desrosiers *et al.*, 1997; Searles *et al.*, 1999). The genomes of these isolates have been fully sequenced (Alexander *et al.*, 2000; Searles *et al.*, 1999). Rhadinoviruses closely related to RRV have been cultured from other macaque species, specifically *Macaca nemestrina* and *Macaca fascicularis* (Auerbach *et al.*, 2000; Mansfield *et al.*, 1999).

Short stretches of related but phylogenetically distinct sequences have also been amplified from *Macaca mulatta* and *Macaca nemestrina* (Bosch *et al.*, 1999; Strand *et al.*, 2000; Schultz *et al.*, 2000). The putative viruses represented by these sequences, named retroperitoneal fibromatosis herpesvirus *macaca mulatta* and *macaca nemestrina* (RFHVMm and RFHVMn, respectively), have been difficult to culture and lack information on genomic organization and sequence relatedness of the complete viral genome. Short stretches of related sequence have also been amplified from African green monkeys (*Chlorocebus aethiops*). In one particular study on rates of infection, 6 of 68 monkeys were found to be infected with *Chlorocebus aethiops* rhadinovirus 1 (ChRV1) while 22 of 68 monkeys were positive for *Chlorocebus aethiops* rhadinovirus 2 (ChRV2) (Greensill *et al.*, 2000b). Thus, the putative viruses represented by these sequences fall into two distinct phylogenetic groupings RV1 and RV2 (Fig. 61.2), neither of which have yet been successfully cultured in vitro (Greensill *et al.*, 2000b). In these studies, PCR products of the DNA polymerase genes from ChRV1, ChRV2, RFHVMm, and RFHVMn were amplified (Rose *et al.*, 1997; Bosch *et al.*, 1999; Strand *et al.*, 2000; Schultz *et al.*, 2000).

The above phylogenetic studies suggested the presence of at least two distinct lineages of KSHV-like rhadinoviruses in primates. One lineage contains KSHV, RFHVMm, RFHVMn, and ChRV1, and the other contains RRV, ChRV2 and *Macaca nemestrina* rhadinovirus type 2 (MneRV2). Short stretches of rhadinovirus sequences have since been amplified from chimpanzees, gorillas and mandrills (Lacoste *et al.*, 2000a,b; 2001; Greensill *et al.*, 2002a). Similar to the two distinct rhadinoviruses found in rhesus macaques and African green monkeys, both mandrills and chimpanzees also contain two gamma-2 herpesviruses, MndRHV1 and MndRHV2 for mandrills and PtRV-1 or PanRHV1a/1b and PanRHV2 for chimpanzees (Greensill *et al.*, 2000a; Lacoste *et al.*, 2000a,b) (Table 61.1, Fig. 61.2).

Retroperitoneal fibromatosis herpesviruses: RFHVMm and RFHVMn

Sequence from the RFHVMm and RFHVMn viruses were amplified from macaques (*Macaca mulatta* and *Macaca nemestrina*) affected with retroperitoneal fibromatosis (RF) (Rose *et al.*, 1997). RF is a vascular fibroproliferative disease that resembles Kaposi's sarcoma (KS) morphologically and histologically (Rose *et al.*, 1997). Nine of 40 *Macaca nemestrina* monkeys with retroperitoneal fibromatosis were positive for RFHvMn by PCR, while two out of two *Macaca mulatta* monkeys with RF were positive for RFHvMm. Two of two unaffected *Macaca nemestrina* monkeys were negative for RFHvMn by PCR (Rose *et al.*, 1997). As described above, phylogenetic analysis demonstrated that these two viruses clustered with KSHV in the RV1 genogroup, while RRV and MneRV2 cluster in a RV2 genogroup. This classification was originally based on the sequence of the viral DNA polymerase gene. To date, only a 7.7 kb fragment of the RFHVMm genomic sequence has been cloned (Rose *et al.*, 2003). This segment of the viral genome codes for glycoprotein B (gB), ORF 9, ORF10, vIL6, vDHFR, K3 and viral thymidylate synthase (TS). These genes are organized in a similar linear fashion as the corresponding homologs in KSHV. However, RFHVMm and RFHVMn are missing ORF11. Analysis of the amino acid similarity between the RFHVMm/RFHVMn and KSHV proteins indicated that RFHVMm and RFHVMn are most closely related to KSHV than any other virus, thus supporting their grouping in the RV1 lineage (Rose *et al.*, 2003). Further comparisons and evolutionary

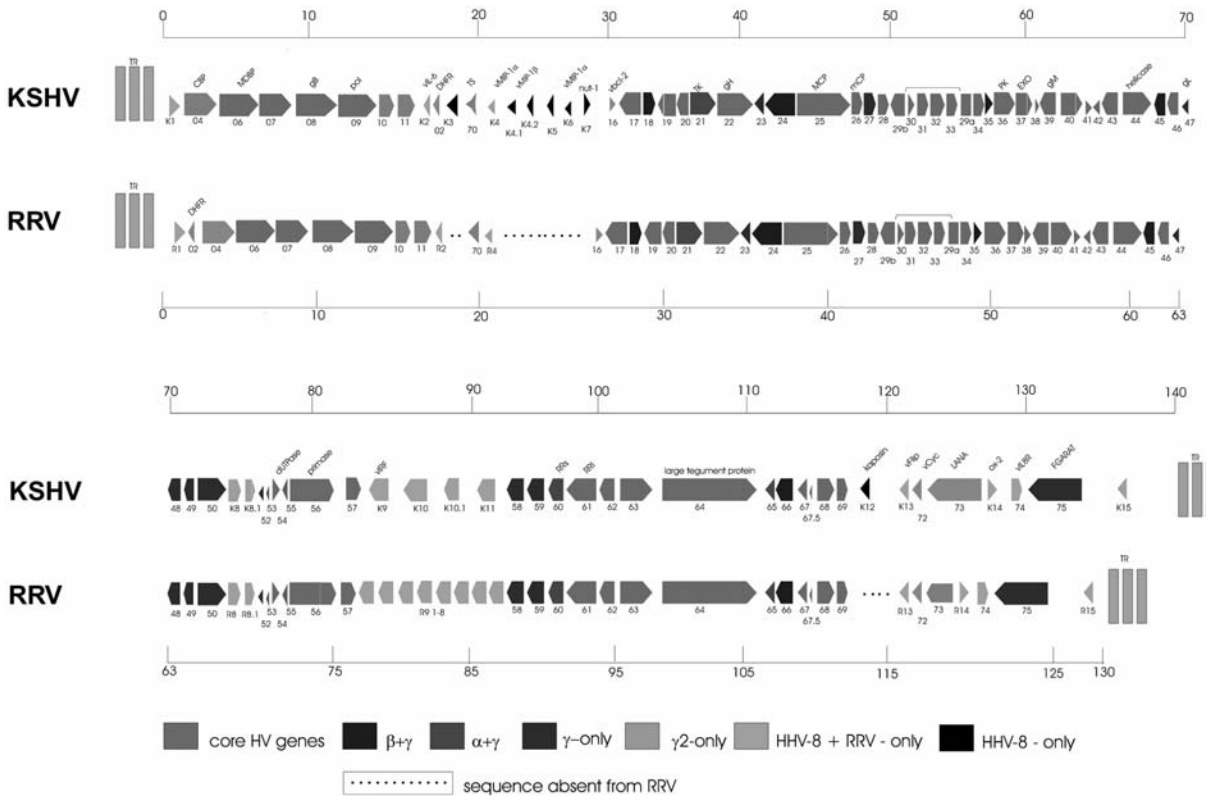

Fig. 61.3. Alignment of the KSHV and RRV genomes. The different colors signify ORFs contained in KSHV and RRV 26–95 that are conserved in the indicated herpesvirus subfamilies or subgroups. The square side of the symbol signifies the 5′ end and the pointed side of the symbol signifies the 3′ end of the depicted ORFs. The ORFs are not drawn to scale. (Taken from Alexander *et al.*, 2000, with permission from the *Journal of Virology*.) (See color plate section.)

analyses await sequencing of the complete RFHVMm and RFHVMn genomes. The RFHVs are currently being amplified from paraffin-embedded or frozen retroperitoneal fibromatosis tumor samples, and are not widely prevalent in the macaque populations housed in most US primate centers.

Rhesus monkey rhadinovirus (RRV)

Greater than 90% of adult macaques at the New England Regional Primate Center (NERPRC) are seropositive for RRV (Mansfield *et al.*, 1999; Desrosiers *et al.*, 1997). A similar frequency of infected macaques was also found at the Oregon Regional Primate Research Center (Wong *et al.*, 1999). Thus, RRV appears to be a natural infectious agent of macaques. However, newborn macaques can be raised free of RRV by hand-rearing (Mansfield *et al.*, 1999). RRV isolated from one species can readily infect macaques of another species upon experimental inoculation (Mansfield *et al.*, 1999). Although gB sequences of RRV isolates from three different macaque species, *Macaca mulatta, Macaca nemestrina* and *Macaca fascicularis* were all closely related, they clustered according to species of origin (Auerbach *et al.*, 2000). A limited study of R1 sequences of RRV from three different species did not reveal a clustering according to species of origin (Damania *et al.*, 1999).

Genomic organization of RRV

Two complete RRV genomes have been sequenced at the Oregon Regional Primate Research Center (ORPRC) and the NERPRC. RRV strain 26–95 from NERPRC and RRV strain 17577 from ORPRC are very similar both in sequence

Table 61.4. A comparison of proteins encoded by the human and macaque rhadinoviruses

RRV (strain 26–95)	% aa similarity to KSHV	Function	Positional homologue in KSHV
R1	33.0%	Unique gene (Transforming gene)	K1
R2/ vIL-6	30.6%	IL-6 homologue	vIL-6
R4/vMIP	42.4%	MIP homologue	vMIP I,II,III
Orf16	58.0%	Bcl-2 homologue	vBcl-2
Orf50/Rta	55.0%	Transcription factor	Orf50/Rta
R8	53.0%	Replication protein	K8/bZip
R8.1	43.0%	Glycoprotein	K8.1
R9.1	60.6%	IRF homologue	vIRF
R13	40.1%	FLIP homologue	vFLIP/Orf71
Orf72	50.2%	Cyclin homologue	vCyclin/Orf72
Orf73	24.9%	Episome maintenance	LANA/Orf73
Orf74	54.7%	G-protein coupled receptor	vGPCR/Orf74
R14	37.6%	N-CAM Ox-2 homologue	K14

and genomic organization (Searles *et al.*, 1999; Alexander *et al.*, 2000). Only four of the 84 ORFs exhibited less than 95% sequence identity at the amino acid level (Alexander, 2000). The primary sequence of the long unique region (LUR) of RRV2695 consists of 130 733 base pairs that contain 84 open reading frames (Fig. 61.3). The overall organization of the RRV genome was found to be very similar to that of human KSHV (Alexander *et al.*, 2000). BLAST search analysis revealed that in almost all cases, RRV coding sequences have greater degrees of similarity to corresponding KSHV sequences than to any other herpesvirus (Table 61.4). All of the ORFs present in KSHV have at least one homologue in RRV except KSHV K3 and K5, K7 (nut-1), and K12 (Kaposin). RRV contains one macrophage inflammatory gene (MIP-1) and eight interferon regulatory factor (vIRF) homologues compared to three MIP-1/vCCLs and four vIRF genes in KSHV. All homologues are correspondingly located in KSHV and RRV with the exception of DHFR. The location of the DHFR gene in KSHV is different from that in RRV and HVS, suggesting that KSHV may have rearranged or reacquired the DHFR gene during the course of evolution from an Old World rhadinovirus to the human rhadinovirus or that RRV acquired the DHFR gene after divergence from KSHV (Fig. 61.3). Only four of the corresponding ORFs between RRV strain 26–95 and RRV strain 17577 exhibit less than 95% sequence identity: these include the glycoproteins H and L genes, uracil DNA glycosidase, and a tegument protein (ORF 67). Analysis of the two genomes indicate that RRV26–95 and RRV17577 are clearly independent isolates of the same virus species and both are closely related in structural organization and overall sequence to KSHV (Searles *et al.*, 1999; Alexander *et al.*, 2000). In addition to homology between the KSHV and RRV ORFs, there is also similarity with respect to the splicing patterns of viral genes. For example, the RRV Orf50, R8 and R8.1 polycistronic transcript is spliced in the same manner as the transcript encoding the corresponding genes in KSHV, (DeWire *et al.*, 2002; Lin *et al.*, 2002) and the LANA, vCyclin, vFLIP transcripts are likewise spliced in a similar fashion (DeWire *et al.*, 2002).

RRV R1

The first open reading frame of RRV encodes for a gene named R1, which is located at an equivalent position in the genome as the K1 gene of KSHV (Damania *et al.*, 1999). Analogous to KSHV K1, R1 is also a transforming gene (Damania *et al.*, 1999). R1 can transform rodent fibroblasts and functionally substitute for STP-C of HVS in immortalizing common marmoset PBMCs to IL-2 independent growth in vitro (Damania *et al.*, 1999). Injection of R1-expressing rodent fibroblasts into nude mice resulted in the formation of multifocal and disseminated tumors in these mice (Damania *et al.*, 1999). Although R1 shows limited sequence homology to K1, the amino-terminal extracellular domains of both K1 and R1 closely resemble members of the immunoglobulin receptor superfamily and exhibit 40% similarity to each other at the amino acid level (Damania *et al.*, 1999; Lee *et al.*, 1998). However, the cytoplasmic tail of R1 is significantly longer than that of K1 and contains several potential SH2 binding motifs, which function as ITAMs to elicit B-cell activation (Damania *et al.*, 2000).

RRV vIL6

KSHV, RRV and RFHV are the only herpesviruses that encode for a viral interleukin 6 homologue (vIL6). RRV vIL-6 has been shown to functionally stimulate the IL-6

receptor/gp130 pathway (Kaleeba *et al.*, 1999). The IL-6R-binding residues in the KSHV and RRV vIL-6 proteins are conserved suggesting that RRV vIL-6 may also bind gp130 in the absence of IL-6R, similar to KSHV vIL-6 (Kaleeba *et al.*, 1999).

RRV vGPCR

KSHV and RRV contain genes for a viral G-protein coupled receptors (vGPCR) that shows homology to the cellular GPCR, IL-8 receptor. Like KSHV vGPCR (for review see Moore and Chang, 2003), RRV vGPCR can transform NIH3T3 fibroblasts in vitro, and stimulate an increased secretion of vascular endothelial growth factor from these cells. RRV vGPCR has been shown to activate the ERK1/2 (p44/42) mitogen-activated protein kinase signaling pathway (Estep *et al.*, 2003). These results suggest that RRV vGPCR, like KSHV vGPCR, may play a role in viral pathogenesis in vivo. The RRV vGPCR transcript is bicistronically expressed with RRV R 15 (Pratt *et al.*, 2005). RRV R15 encodes a viral CD200 protein which shares homology with human and KSHV CD200 proteins (Langlais *et al.*, 2006).

RRV Orf50/Rta and RRV R8

The KSHV Orf50/Rta transactivator is encoded by a polycistronic transcript, which also contains the multiply spliced KSHV K8 and K8.1 genes. Analogous to the situation with KSHV, the homologous genes in RRV, Orf50/Rta, R8 and R8.1, respectively, are also encoded by a polycistronic, spliced transcript (DeWire *et al.*, 2002; Lin *et al.*, 2002). The KSHV Orf50 mRNA has a small 5′ exon, followed by a larger second exon encoding the remainder of the Orf50 sequence (Zhu *et al.*, 1999). Similarly in RRV, the Orf50 mRNA has a small upstream exon followed by a larger second exon (DeWire *et al.*, 2002; Lin *et al.*, 2002). In contrast, the gamma-1 herpesvirus, EBV, encodes an Orf50 homolog, BRLF1, whose cDNA is identical to the genomic ORF (Manet *et al.*, 1989). The RRV Orf50 protein is localized to the nucleus and functions as a transcriptional activator (DeWire *et al.*, 2002). A striking observation is that the KSHV Orf50 protein can activate certain RRV promoters, while the RRV Orf50 protein can also activate a subset of KSHV promoters, suggesting that there is a conservation in protein function of the Orf50/Rta transactivators of these rhadinoviruses (DeWire *et al.*, 2002; Damania, 2004).

Unrecognizable from the RRV genomic sequence alone, two other spliced genes of RRV include the R8 and R8.1 genes. The R8 protein is localized in the nucleus and shows 39% identity and 53% similarity at the amino acid level to the K8/bZip protein of KSHV. The R8.1 protein is localized in the cytoplasm and shows 26% identity and 43 % similarity at the amino acid level. The R8.1 protein has two potential glycosylation sites (NXS/T) (DeWire *et al.*, 2002).

RRV LANA

RRV also encodes a latency-associated nuclear antigen (R-LANA). Similar to KSHV LANA, R-LANA exhibits a nuclear speckled localization and possesses the ability to homodimerize. R-LANA can inhibit viral lytic replication by repressing the transactivation function of RRV Orf50/Rta. The mechanism for this repression involves the recruitment of histone deacetylase complexes since R-LANA′s ability to repress RRV Orf50 transactivation was reversed by the addition of the HDAC inhibitor trichostatin A (TSA) and TSA could significantly reactivate RRV from latently infected cells (Dewire and Damania, 2005).

RRV transcription program

The overall transcription profile of the RRV lytic cycle is very similar to that of KSHV. The immediate early genes expressed during the RRV lifecycle are Orf50 and Orf57, similar to the case seen with KSHV reactivation. Consistent with the KSHV transcriptome, early genes transcribed by RRV include vIL6, DNA polymerase, and R1 (DeWire *et al.*, 2002; Dittmer *et al.*, 2005). An exception to this pattern is the cluster of genes classically defined as latent in KSHV, i.e., Orf73 (LANA), vCyclin, and vFLIP, which are expressed as delayed early genes during the RRV lytic cycle (DeWire *et al.*, 2002). Late transcripts include those of RRV vIRF-1, Orf62, Orf65, and gB genes (DeWire *et al.*, 2002; Dittmer *et al.*, 2005).

RRV capsid structure

As with other herpesviruses, RRV lytic infection of rhesus fibroblasts leads to the synthesis of three distinct intranuclear capsid species. A and B capsids do not contain viral genomes, while C capsids do contain the viral genome at their center and are considered infectious. There are multiple similarities in capsid structure and assembly between KSHV and RRV. Both viruses make all three types of intranuclear capsids (A, B and C) during lytic infection, and the three RRV capsid species are similar in structure to KSHV as measured by transmission electron microscopy (TEM) (O'Connor *et al.*, 2003). In addition, both the RRV and KSHV virions assemble with similar slow kinetics and the capsids possess a highly conserved protein composition (O'Connor *et al.*, 2003). Both the RRV and KSHV capsid proteins include the major capsid protein (MCP) encoded by ORF25,

components of the triplex, TRI-1 and TRI-2, encoded by RRV ORFs 62 and 26 respectively, RRV SCIP encoded by ORF65, and RRV SCAF encoded by ORF 17.5 (O'Connor *et al.*, 2003). All five RRV capsid proteins demonstrate similarity to their KSHV homologues at the amino acid level (O'Connor *et al.*, 2003; Alexander *et al.*, 2000; Searles *et al.*, 1999). However, one difference between these viruses is the efficiency of capsid assembly. As opposed to the inefficiency of KSHV maturation following reactivation from latently infected B-cell lines (Nealon *et al.*, 2001), de novo RRV infection results in the release of much higher levels of infectious virions with genome-containing C capsids at their center and a lower amount of the incomplete A and B capsids which do not contain viral genomes (O'Connor *et al.*, 2003). This discrepancy may explain the reason why RRV can be grown to high titers ($\sim 10^6$ pfu/ml) (DeWire *et al.*, 2003) in vitro relative to KSHV, since only virions containing intact viral genomes (i.e. C capsids) are infectious (O'Connor *et al.*, 2003). Three-dimensional structure comparisons of the RRV capsid structure by electron cryomicroscopy have shown that the A capsid is empty and its penton channels are open, the B-capsid contains a scaffolding core and its penton channels are closed, and the C-capsid contains a DNA genome closely packed as regularly spaced density shells (25 Å apart), and its penton channels are open (Yu *et al.*, 2003). The RRV A capsid reconstruction was achieved at 15-Å resolution (Fig. 61.4), which is the best, achieved for any gammaherpesvirus to date. The best resolution for the KSHV capsid was achieved at 24-Å and both capsids appeared almost identical (Yu *et al.*, 2003; Wu *et al.*, 2000). Further, although the RRV capsid showed overall structural similarities to alpha and betaherpesvirus capsids, there were prominent differences in the Ta triplex and SCIP structures suggesting that SCIP and triplex, together with tegument and envelope proteins, confer structural, and potentially functional, specificity to alpha-, beta- and gamma-herpesviruses (Yu *et al.*, 2003) (Fig. 61.4). Thus, this study validates the use of RRV as a model system for the study of KSHV and other gammaherpesviruses, since the latter appear significantly different from the alpha and beta herpesviruses, despite the fact that the individual structural proteins of all three groups of herpesviruses share a high degree of homology.

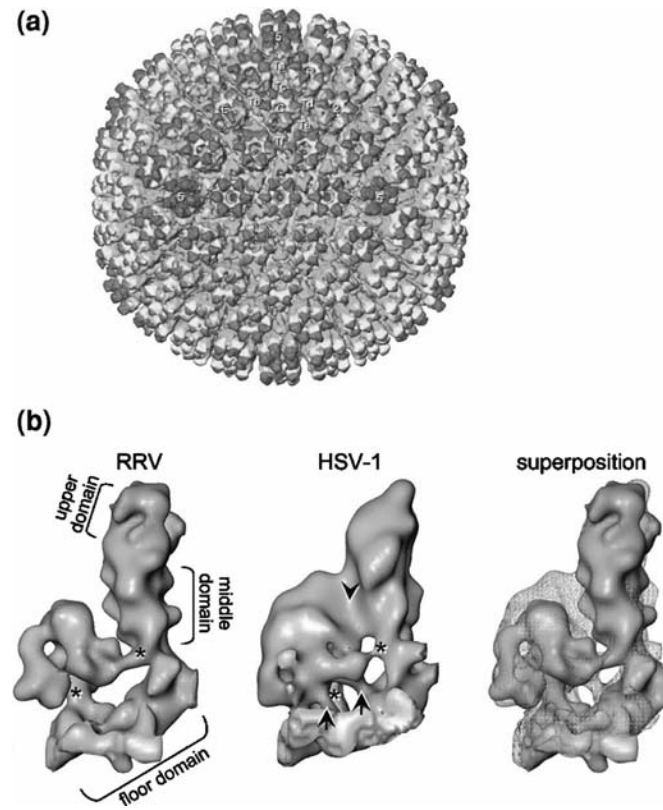

Fig. 61.4. Structural similarity between the KSHV and RRV capsids. (a) The RRV A capsid structure at 15 Angstrom resolution. (b) Structural difference between the gammaherpesvirus RRV and alphaherpesvirus HSV-1 capsids. The figure represents one penton sub-unit and one triplex (Ta) that was computationally extracted from the RRV A capsid taken at 15 Angstroms shown in panel A, and an HSV-1 capsid map filtered to the same resolution. The RRV and HSV-1 Ta triplex interacts with MCP through the leg connected to the MCP floor domain, as well as through a small link to the MCP middle domain (indicated by asterisks). The differences between the RRV and HSV-1 capsids are indicated by an arrowhead and arrows, and represent interactions that are present in HSV-1 but missing in RRV.
(Taken from Yu *et al.*, 2003, with permission from the *Journal of Virology*.)

RRV pathogenesis

Wong *et al.* (1999) at the ORPRC experimentally inoculated rhesus macaques with RRV (strain 17577) with and without SIVmac239 co-infection to determine whether RRV played a role in the development of lymphoproliferative disease. In contrast to two control animals inoculated with SIVmac239 or RRV alone, two of two animals coinfected with SIVmac239 and RRV17577 developed hyperplastic lymphoproliferative disease resembling the multicentric plasma cell variant of Castleman's disease, characterized by persistent angiofollicular lymphadenopathy, hepatomegaly, splenomegaly and hypergammaglobulinemia. Hypergammaglobulinemia was associated with severe immune-mediated hemolytic anemia in one RRV17577/SIV-infected macaque. Thus, experimental RRV17577 infection of

SIV-infected rhesus macaques was associated with the hyperplastic B cell lymphoproliferative manifestations analogous to those seen in individuals infected with KSHV. Mansfield *et al.* (1999) at the NERPRC have also performed co-infections using RRV (strain 26–95) and SIV mac239. Experimental infection of macaques was associated with a lymphadenopathy that was characterized by paracortical hyperplasia and vascular hypertrophy/hyperplasia that subsequently was replaced by marked follicular hyperplasia. In the most severe cases, this follicular hyperplasia destroyed the medullary sinuses and completely effaced the normal lymph node architecture. Similar changes have been found in KSHV infected, HIV-negative human patients with histologic features of angioimmunoblastic lymphadenopathy and reactive lympadenopathy. B-cell proliferation is a feature common to multicentric Castleman's disease and angioimmunoblastic lymphadenopathy (Mansfield *et al.*, 1999). However, 12 weeks post-RRV infection, these pathologies appeared to be resolved. Thus, the immune system in these macaques was able to halt the progression of the hyperplasia. In addition, Mansfield *et al.* (1999) observed that three of four monkeys co-infected with RRV and SIV developed an arteriopathy. This arteriopathy was similar to the vascular endothelial lesion seen in patients with KS and to the large vessel arteritis in MHV68 infected mice. Despite some drawbacks to the RRV model system, a large number of critical questions can still be addressed using this system of experimental infection.

RRV as an animal model system for KSHV

Since its discovery in 1994, studies to identify a KSHV lytic culture system have been intense. Although lytic systems for KSHV have been developed in several laboratories using reactivated B-cells, 293 cells and *de novo* infection of endothelial cell lines (Renne *et al.*, 1998; Lagunoff *et al.*, 2002; Poole *et al.*, 2002; Cannon *et al.*, 2000, Sun *et al.*, 1998; Moses *et al.*, 1999; Foreman *et al.*, 1997), these systems yield low quantities of virus resulting in limited replication and serial transmission. The lack of a traditional permissive system for KSHV limits the ability to study the temporal order of events of the full virus life cycle. There have been two KSHV bacterial artificial chromosomes (BAC) constructed to date (Zhou *et al.*, 2002; Delecluse *et al.*, 2001). However, methods to analyze properties of recombinant KSHVs are limited. In contrast, RRV can be grown to high titers ($\sim$1x10^6 pfu/ml) in rhesus fibroblasts (RhFs) (DeWire *et al.*, 2003) and both plaque assays and real-time PCR assays to measure RRV replication have been devised (DeWire *et al.*, 2003). In addition, RRV can be used as a genetic system to create recombinant RRVs as exemplified by the construction of a recombinant RRV virus expressing green fluorescent protein (RRV-GFP)(DeWire *et al.*, 2003). An infectious RRV model system has been developed such that naïve RRV-negative rhesus macaques inoculated with RRV demonstrate persistent viral infection (Mansfield *et al.*, 1999). Use of the rhesus virus can help address a large number of critical questions. These include the ability to define the relative importance and contribution of individual genes to the establishment of primary infection, dissemination, persistence, tropism, pathologic manifestations and immune avoidance strategies.

At the present time, RRV is the closest simian KSHV homologue that can be grown lytically and has a genetic system to analyze viral gene function. The transcription program of RRV resembles that of KSHV (DeWire *et al.*, 2002), the capsid structure and assembly of RRV and KSHV virions are very homologous (O'Connor *et al.*, 2003; Yu *et al.*, 2003) and a method to generate RRV recombinant viruses has been established (DeWire *et al.*, 2003). Thus, the biological properties of RRV, the ability to construct recombinant RRVs and the ability to generate persistent RRV infection in naïve rhesus macaques experimentally infected with RRV, makes RRV a tractable model to study the KSHV lifecycle in vitro and in vivo.

Conclusions

The presence of gammaherpesviruses in most primates tested to date alludes to the fact that these viruses evolved with their host species. They share similarity in genomic sequence, organization and biological properties. Current evidence indicates that there are two distinct lineages of primate rhadinoviruses and lymphocryptoviruses, both of which are evolutionarily related to KSHV. This suggests that the gammaherpesviruses have diversified from a common ancestor in a manner mediating co-speciation of herpesviruses with their host species. As this theme is conserved for all gammaherpesviruses of Old World Primates including the great apes, it raises the possibility that viruses belonging to a second major phylogenetic grouping of lymphocryptoviruses and rhadinoviruses may be found in humans. The evidence suggests the possible existence of a novel genogroup 2 human lymphocryptovirus which include viruses like GgorLCV2 and PpygLCV1 (Ehlers *et al.*, 2003), and a novel human rhadinovirus belonging to the RV2 genogroup, which include viruses like RRV and PanRHV2 (Lacoste *et al.*, 2000a, 2001).

The non-human primate viruses provide a means to model human infection in an experimental setting that

would not be possible in humans. While use of the simian viruses as animal models for human EBV and KSHV are still in the early developmental stage, they will likely prove useful for studying the pathogenesis, prevention, and treatment of gammaherpesvirus infections.

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Part V

Subversion of adaptive immunity

Edited by Richard Whitley and Ann Arvin

62

Herpesvirus evasion of T-cell immunity

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The multiple layers of the human immune response present a challenge to viruses, which must survive and multiply within a host for a sufficient period of time to allow successful transmission to susceptible individuals. Given the large proteomes and comparatively low polymerase error rate of human herpesviruses, antiviral immunity at first glance appear to have the upper hand. Nonetheless, herpesviruses manage prolonged incubation periods following initial infection, with systemic dissemination and prolonged secretion, often from multiple sites. In contrast to the similarly large poxviruses, the ability to subsequently establish persistent infection is a hallmark of the human herpesviruses. To enable this lifestyle, the herpesviruses devote a significant proportion of their genome coding capacity to the expression of immuno-evasins, a collection of molecules that disrupt normal immune physiology. Each human herpesvirus studied has evolved elegant cell biological solutions to problems posed by the immune response.

Innate immunity, an evolutionarily conserved and relatively non-specific system of pattern recognition molecules hardwired in the genome, cytokines such as interferons, phagocytes and natural killer (NK) cells, represents the first line deployed against microbial invaders, including herpesviruses (Janeway and Medzhitov, 2002). The clonal expansion of B- and T- lymphocytes that bear antigen-specific receptors for viral epitopes underlies the adaptive antiviral immune response, laying the groundwork for a highly pathogen-specific defense. Such specificity comes at a price – lymphocyte proliferation requires time to unfold, and innate immunity, in particular NK-cell activity, limits the initial herpesvirus spread. Indeed, NK cell immune deficiencies result in dramatic infection by several herpesviruses (McClain *et al.*, 1988; Biron *et al.*, 1989). There is significant cross-talk between the innate and adaptive systems, and preliminary pathogen recognition by the innate immune system directly contributes to the development of adaptive immunity. Further, the eventual adaptive response utilizes branches of the innate system for crucial effector function (Medzhitov and Janeway, 1999).

Innate and adaptive immunity act in concert to allow recovery from acute herpesvirus infection. Adaptive immunity then allows for lifelong immunological memory, affording both control of persistent herpesvirus infection and protection against reinfection. Once present, virus-specific CD4+ T-lymphocytes then coordinate the adaptive antiviral response, directing the production of virus-specific immunoglobulin by B-lymphocytes, the antiviral activity of CD8+ T-lymphocytes and NK cells, and further stimulating the activity of phagocytic cells.

Through millennia of coevolution, herpesviruses have largely reached a state of equilibrium with their human hosts. At the cost of a large proportion of their coding capacity, herpesviruses perturb adaptive immunity to achieve persistent infection, in general with remarkably little collateral damage to their hosts. However, lapses in T-cell immunity, such as by immunosuppressive agents or by coinfection with other pathogens such as Human Immunodeficiency Virus, can lead to significant herpesvirus-associated pathology.

Human herpesvirus genome size and polymerase fidelity place constraints on epitope mutation, and generally do not allow for antigenic variation as a means to avoid T-cell immunity. Herpesviruses therefore, have devised a range of mechanisms to subvert adaptive immunity. Generalized T-cell immuno-evasion strategies shared by herpesviruses include latency, restriction of viral gene expression to immunoprivileged sites such as the CNS, interference with complement, cytokines, NK-cell function, and apoptosis,

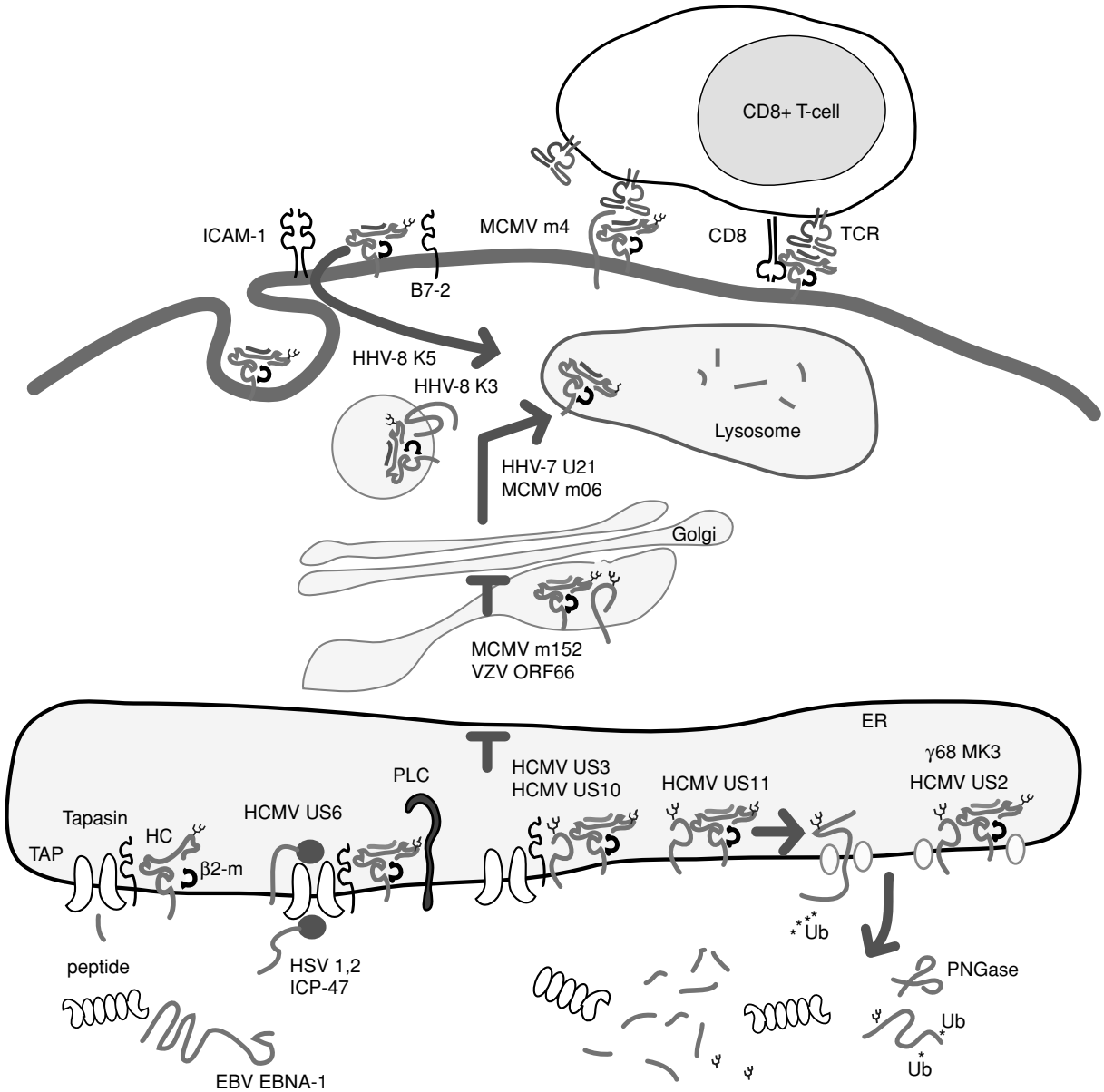

Fig. 62.1. Herpesvirus immunoevasins that directly interfere with class I MHC molecule biosynthesis. Class I MHC molecules are assembled from free HC and β-2 microglobulin within the ER, along with antigenic peptide. Peptides are produces by cytosolic proteasome degradation. The EBV EBNA-1 GAr domain interferes with proteasomal degradation in-*cis*. Tapasin and the PLC facilitate loading of peptide cargo onto empty class I MHC molecules. HSV-1,2 ICP47 and HCMV US6 block TAP peptide transport, while HCMV US3 inhibits tapasin and retains class I complexes in the ER. Following receipt of peptide, the loaded class I MHC molecules travel through the secretory pathway to the cell surface. HCMV US10 delays transport of class II molecules from the ER, while VZV ORF66 and MCMV m152 retain class I MHC in the Golgi complex. HCMV US2, US11 and MHV-68 MK3 dislocate class I molecules via an unidentified ER membrane pore to the cytosol. The dislocated class I MHC heavy chains are ubiquitinated (Ub) and deglycosylated by cellular PNGase prior to proteasomal cleavage. HHV-7 U21, MCMV m6 and HHV-8 K3 redirect class I molecules from the secretory to the endolysosomal pathway for degradation. HHV-8 K5 likewise targets class I MHC, B7-2 and ICAM-1 molecules to the endolysosomal pathway for destruction. MCMV m4 disrupts recognition of cell surface-disposed class I MHC molecules by CD8+ T-cells. (See color plate section.)

all of which are reviewed in detail in other chapters. This section will highlight the cell biology that underlies herpesvirus evasion of T-cell immunity.

Disruption of class I MHC antigen presentation

Presentation of virus-derived antigenic peptides by major histocompatibility (MHC) class I molecules plays a key role in the clearance of many viruses from the body. Viral proteins expressed in the cytosol may be targeted for degradation and presented by class I MHC, alerting CD8+ T-cells to the presence of an intracellular viral pathogen. CD8+ lymphocytes bear the T-cell receptor (TCR), which recognizes peptides in an MHC-restricted fashion. Recognition of peptide cargo derived from foreign proteins alerts the CD8+ T-cell, and in the proper context stimulates cytotoxic T-cell (CTL) mediated lysis of the infected target cell. Though sacrificing the host cell, lysis ultimately limits the spread of the viral pathogen (Heemels and Ploegh, 1995). Interference with the class I pathway appears to be a central mechanism of immuno-evasion of persistent viruses, herpesviruses in particular (Tortorella *et al.*, 2000). All human herpesviruses examined thus far employ strategies to perturb class I MHC antigen presentation to CD8+ T-cells (Fig. 62.1, Table 62.1).

Class I MHC molecules are heterotrimeric complexes that consist of a heavy chain, light chain and antigenic peptide (Heemels and Ploegh, 1995). The class I MHC heavy chain is a 43-kDa polymorphic type I membrane glycoprotein that binds antigenic peptide cargo, generally of 8–10 residues in length. The heavy chain α1 and α2 domains form the peptide-binding groove, consisting of two antiparallel α-helices and an eight-stranded β-sheet (Bjorkman *et al.*, 1987). The class I MHC light chain, or β-2 microglobulin, is an invariant 12-kDa protein of the immunoglobulin (Ig) fold family.

Class I MHC peptide ligands are primarily generated in the cytosol by the 26S proteasome, a barrel-shaped multicatalytic threonine protease complex whose 20S core is composed of α and β subunits. The β subunits, x, y and z, contain the protease active sites with tryptic, chymotryptic and caspase-like protease activities, respectively (Voges *et al.*, 1999). Upon Interferon-γ (IFN-γ) stimulation, LMP-2, LMP-7 and MECL-1 replace the x, y, and z subunits, respectively, giving rise to the immunoproteasome. IFN-γ also induces expression of the 11S proteasome regulator PA28αβ. The mammalian MHC complex encodes the LMP2 and LMP7 subunits (Brown *et al.*, 1991). While the proteasome and immunoproteasome have substrate specificity, the immunoproteasome may generate peptides with motifs suitable for class I MHC presentation at higher frequency (Nazif and Bogyo, 2001).

Interference with proteasomal proteolysis

Both human CMV (HCMV) and murine CMV (MCMV) can disrupt immunoproteasome formation (Khan *et al.*, 2004). MCMV appears to achieve this phenotype via its M27 gene-product. M27 targets STAT2, a key molecule in the IFN-γ signal transduction pathway, for degradation by the ubiquitin-proteasome pathway. Deletion of M27 restores fibroblast immunoproteasome formation and enhances sensitivity to IFN-γ in cell culture (Khan *et al.*, 2004). MCMV mutants that lack functional M27 activity are attenuated in growth and virulence (Abenes *et al*, 2001), a phenotype that is partially restored in IFN-γ receptor knockout mice (Zimmermann, 2005). M27 is conserved in all γ-herpsevirinae; the homologous HCMV UL27 remains poorly characterized, though HCMV UL27 deletion mutants likewise display attenuated growth in culture and *in vivo* (Prichard *et al.*, 2006).

EBV and CMV encode factors that disrupt proteasomal proteolysis of specific substrates. Vigorous CD8+ T-cell responses can be detected against all EBV nuclear antigens, except EBNA-1, which eludes T-cell recognition (Khanna *et al.*, 1995). EBNA-1 is essential for viral genome persistence during cell division and is a major protein expressed during latency (Lee *et al.*, 2004). Although EBNA-1 specific CD8+ T-cells can be isolated from peripheral blood of EBV-infected individuals, they are less abundant than T-cells specific for other EBV nuclear antigens. Further, EBNA-1-specific T-cells generally fail to be activated during EBV infection (Khanna *et al.*, 1995; Blake *et al.*, 1997). This stealth behavior has been attributed to the unusually long EBNA-1 half-life, enabled by the presence of its internal Gly–Ala repeat domain (GAr). The GAr domain is reported to act as a *cis*-inibitor of ubiquitin-proteasomal proteolosis in B cells, and thereby prevents the introduction of EBNA peptides into the class I pathway. The GAr domain is both necessary and sufficient for altering protein half-life, and cytosolic fusion proteins that harbor GAr motifs gain resistance to proteasomal proteolysis (Dantuma *et al.*, 2002). For instance, fusion of a 200-residue or even 17-residue Gly–Ala repeat to EBNA-4 confers resistance to proteasome degradation and results in reduced antigen presentation of EBNA-4 epitopes (Dantuma *et al.* 2002). Several mechanisms have been proposed to explain the inhibitory function of the GAr domain, for instance the formation of tightly-folded beta-sheet structures that resist disassembly and/or interactions with the ubiquitin-proteasomal pathway. GAr also reduce the rate of EBNA-1

Table 62.1. Human herpesvirus immuno-evasins of adaptive T-cell immunity discussed in the chapter

Virus	Immuno-evasin	Putative mechanism
HSV 1/2		
	ICP47	Blocks TAP peptide transport
	Unknown	CD83 internalization and inhibition of DC function.
	Unknown	Inhibition of DC cell maturation
	US1	Kinase activity inhibits CD4+ T-cell activation by B cells
	US3	Kinase activity inactivates (stuns) CTLs in *trans*
	Unknown	Intracellular redistribution of class II MHC molecules
VZV		
	?ORF66	Retains class I in the Golgi complex
	Unknown	Inhibition of DC function, down-regulation of multiple surface proteins
	Unknown	Reduction of cell surface ICAM-1 (CD54)
	Unknown	Inhibition of Jak/STAT expression, disruption of IFN-γ signaling
EBV		
	EBNA-1	Gly-Ala repeat domain blocks proteasomes in *cis*
	BCRF1	IL-10 homologue, down-regulates class I expression
	General	Anchor residue mutations prevent epitope presentation by subset of class I alleles (epitope loss)
	Gp25-gp42-gp85	Inhibits DC maturation
	Unknown	Inhibition of monocyte phagocytic function
	LMP-2A	Activates env superantigen, may dysregulate CD4+ T cell responses
	gp42	Inhibits class II MHC recognition by $CD4^+$ T-cells
CMV		
	Pp65 (UL83	Kinase activity blocks proteasomal proteolysis of IE-1
	US2	Targets folded class I and class II molecules for proteasome degradation
	US3	Inhibits tapasin, retains folded class I and class II molecules in the ER
	US6	Blocks TAP peptide transport
	US8	Binds free class I heavy chains, function unknown
	US10	Delays egress of folded class I molecules from ER
	US11	Targets class I heavy chains for proteasome degradation via a Derlin-1 pathway
	UL18	class II homologue, binds to LIR-1 (ILT-2) inhibitory receptor on APCs, lymphocytes
	UL111A	IL-10 homologue, downregulates class I expression
	UL111.5A	IL-10 homologue, expressed during latency
	M27, ?UL27	Targets STAT2 for degradation, impairs immunoprotasome formation
	Unknown	Interference with DC activity and expression of multiple DC surface proteins, including CD83
	UL20	TCR-γ chain homologue, function unknown
	Unknown	Targets Jak1 for proteasomal degradation, disrupts IFN-γ upregulation of class II MHC expression
	Unknown	Alters trafficking of class II MHC molecules
HHV-6		
	Unknown	Down-regulation of TCR/CD3 expression
HHV-7		
	U21	Targets class I for lysosomal degradation
HHV-8		
	K3/MIR1	Induces rapid internalization of cell surface class I
	K5/MIR2	Induces rapid internalization of cell surface class I, ICAM-1, and B7-2, and PE-CAM

translation (Dantuma *et al.*, 2002). Nonetheless, EBNA-1 peptides can circumvent GAr blockade at a low level via defective ribosomal products (DriPs), misfolded polypeptides that result from errors in translation or folding. DriPs are rapidly degraded and may contribute a significant percentage of all peptides to the class II pathway. Thus, DriPs that do not contain functional GAr sequences should be susceptible to proteasomal degradation, and CD8+ T-cell recognition of EBNA-1 derived peptides have now been described (Lee *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004).

The HCMV matrix phosphoprotein 65 (pp65) can disrupt antigen presentation of epitopes derived from immediate-early protein 1 (IE1) in *trans*. The pp65 kinase can

phosphorylate IE1, and through an unknown mechanism, prevent the proteasomal degradation of IE1 (Gilbert *et al.*, 1996).

Interference with peptide transport

Empty class I MHC molecules receive their peptide cargo shortly after assembly in the endoplasmic reticulum (ER). Empty class I molecules are generally short-lived, and are either retained within the ER or dissociate (Heemels and Ploegh, 1995). Thus, antigenic peptides must be pumped across the ER membrane to reach nascent class I complexes, and this process represents a key control point in the class I MHC biosynthetic pathway. Peptide transport is accomplished by a dedicated MHC-encoded ATP-binding cassette (ABC) family member, the transporter associated with antigen presentation (TAP) (Neefjes *et al.*, 1993). TAP molecules are heterodimers formed by the association of TAP-1 and TAP-2 subunits, which together comprise the peptide-binding site. Peptide substrates, optimally 8–12 residues in length, interact with TAP in two distinct phases: ATP-independent binding; subsequent ATP-dependent peptide transport versus rapid dissociation from TAP (Heemels and Ploegh, 1995).

Several human herpesviruses have convergently evolved polypeptides that inhibit TAP peptide transport. HSV-1 and HSV-2 encode a cytosolic 9-kDa (88 residues HSV-1; 86 residues HSV-2) immediate early gene product, called infected-cell protein 47 (ICP47) (York *et al.*, 1994; Hill *et al.*, 1995). The HSV-1 and HSV-2 ICP47 molecules demonstrate 47% amino acid sequence identity and appear to utilize the same mechanism. ICP47 of both HSVs binds to TAP with high affinity (KD = 50 nM) at a site that overlaps the peptide binding site of both TAP subunits, and functions as a competitive inhibitor of TAP-peptide interactions (Ahn *et al.*, 1997; Tomazin *et al.*, 1998; Pfander *et al.*, 1999). 100- to 1000-fold molar excess of peptide is required to overcome ICP47 inhibition of TAP peptide-transport (Tomazin *et al.*, 1998). ICP47 functions in a species-specific fashion and inhibits human, but not murine, TAP. Residues 3–34 of ICP47 are sufficient to inhibit TAP transport, even when a synthetic 32-mer is added to permeablized cells (Galocha *et al.*, 1997; Neumann *et al.*, 1997; Tomazin *et al.*, 1998).

Preliminary nuclear magnetic resonance structural characterization suggests that ICP47 undergoes a conformational change from an unstructured state in solution to the formation of a helix-turn-helix motif in the presence of a lipid environment (Pfander *et al.*, 1999). The lipid environment greatly increases binding affinity of ICP47 with TAP, where ICP47's amino-terminal helix serves as a membrane anchor (Aisenbray, 2006). How ICP47 itself avoids transport and ATP-induced dissociation remains unclear, but it may rely on interactions outside the TAP peptide binding site to stabilize its cytosolic localization (Galocha *et al.*, 1997). Further understanding of ICP47 function requires structural analysis of the TAP-ICP47 complex.

Apparently despite 100-fold lower affinity for murine TAP, ICP47 deletion mutants nonetheless exhibit reduced neurovirulence in a murine infection model, a phenotype that required the presence of CD8+ T-cells (Goldsmith *et al.*, 1998). Akin to its use by HSV, ICP47 may also at some point be used in clinical applications. Indeed, several groups have explored ICP47 immuno-evasion in applications ranging from gene therapy to xenotransplantation (Berger *et al.*, 2000; Crew and Phanavanh, 2003; Radosevich *et al.*, 2003).

HCMV likewise disrupts TAP transport, though by a distinct approach. The HCMV US genome region encodes a cluster of immuno-evasins that comprise the US2, US6 and US11 glycoprotein families (the US2–US11 gene products) (Britt and Mach, 1996). At least six of these ten gene-products are now known to interact with the class I MHC biosynthetic pathway. HCMV US6 is a 21-kD ER-resident type I integral membrane glycoprotein that is expressed during the late phase of viral infection. US6 interferes with TAP by binding with micromolar affinity to the ER-lumenal face of the transporter. The ER-lumen domain of US6 is necessary and necessary for TAP inhibition (Ahn *et al.*, 1997; Kyritsis *et al.*, 2001). US6 does not appear to function as a competitive inhibitor of TAP peptide transport, and US6 neither influences the amount of peptides that bind to TAP nor the TAP-peptide affinity (Kyritsis *et al.*, 2001). Rather, US6 reduces TAP1 affinity for ATP and may thereby cut off the energy source for peptide transport (Hewitt *et al.*, 2001).

How do interactions with the ER lumen between US6 and TAP influence the cytosolic nucleotide-binding domain? Though presently awaiting experimental confirmation, US6 likely induces a conformational rearrangement that is transmitted to the TAP cytoplasmic ATP-binding site (Hewitt *et al.*, 2001; Kyritsis *et al.*, 2001). Indeed, US6 affects the conformational stability of TAP. In the presence of either US6 or ATP, TAP remains stable at 37 °C, whereas the isolated heterodimer would normally dissociate (Hewitt *et al.*, 2001). Taken together, US6 appears to either lock TAP in a conformational intermediate of its catalytic cycle, or may induce an aberrant conformation. Similarly, recombinant HSV engineered to express other class I MHC immunoevasins cause increased viral burden in the CNS (Orr, 1995). Additional herpesvirinae-encoded TAP inhibitors have recently been discovered, including Epstein–Barr BNLF2a (Ressing, 2005 and Wiertz, E.J., personal communication).

Herpesviruses encode multiple cytokines (virokines) that alter antiviral immunity (reviewed in other chapters). Both EBV and CMV encode homologues of interleukin-10 (IL-10) that retain some, but not all IL-10 functions. Both EBV and CMV IL-10 suppress class I surface levels *in vitro*, including via downmodulation of TAP1 expression and therefore ER peptide transport (Zeidler *et al.*, 1997; Spencer *et al.*, 2002; Jenkins *et al.*, 2004).

Interference with ER chaperones required for class I biosynthesis

Class I MHC molecules are assembled in a multistep process. ER chaperones, including the lectins calreticulin and calnexin, and the thioreductase ERp57 facilitate folding of nascent class I molecules (Cresswell, 2000). The heavy chain/β-2 microglobulin heterodimer is then recruited to the peptide loading complex (PLC), a multiprotein assembly that that includes TAP and facilitates peptide loading (Cresswell, 2000). The ER-resident molecule tapasin is uniquely dedicated to class I molecule assembly. Tapasin tethers empty class I molecules to TAP and also possesses peptide exchange activity, optimizing the class I peptide repertoire by selecting for high affinity class I-peptide complexes (Momburg and Tan, 2002).

The human cytomegalovirus has evolved a mechanism to subvert the interaction between tapasin and empty class I molecules that await peptide cargo. US3, expressed during the immediate-early period of HCMV infection, is 23-kDa type I integral membrane capable of oligomerization (Jones *et al.*, 1996; Misaghi *et al.*, 2004). US3 binds directly to both class I molecules and tapasin, and causes ER-retention of class I via two mechanisms. First, the US3 ER lumenal domain contains a retention sequence that is distinct from the canonical KDEL or KKXX ER-retention motifs. The US3 residues Ser-58, Glu-63, and Lys-64 are required for ER-retention and may interact with an ER-resident protein, such as tapasin (Lee *et al.*, 2003; Misaghi *et al.*, 2004). US3 may retain class I molecules in the ER via direct association, in effect physically preventing class I molecules from traveling along the secretory pathway to the cell surface (Lee *et al.*, 2003; Misaghi, *et al.*, 2004). Interestingly, both the US3 ER-lumenal and transmembrane domains are required to bind class I molecules, and the affinity of class II binding appears to correlate with the extent of ER retention for certain alleles (Lee *et al.*, 2000; Park *et al.*, 2004). NMR structural analysis and comparison with the homologous CMV US2 gene-product (see below) suggests that the US3 ER-lumenal domain forms an Ig-like fold. Though Ig-like folds underlie many protein–protein interactions, even when present at high concentrations, the US3 ER-lumenal domain by itself does not appear to interact with class I with any measurable affinity in vitro or in vivo (Lee *et al.*, 2000; Misaghi *et al.*, 2004). Thus, the transmembrane domain does not appear to simply increase US3 local concentration by confining it to the plane of the ER membrane. Although the structural mechanism by which both the soluble and transmembrane domains are required for class I binding remains unclear, interactions within the lipid bilayer underlie the activity of several herpesvirus class I immuno-evasins.

US3 has a short half-life and associates with class I molecules only transiently. Perhaps the dominant mechanism of US3 function instead results from its direct inhibition of tapasin-dependent peptide loading (Park *et al.*, 2004). Reminiscent of US6 activity, US3 may induce a conformational change in tapasin and thereby alter tapasin-induced peptide loading and exchange activity. A subset of class I alleles do not require tapasin for peptide loading and surface expression, and these class II molecules largely escape US3-mediated ER retention (Park *et al.*, 2004). An alternatively spliced form of US3 lacking the transmembrane domain can associate with tapasin, but not class I MHC molecules, and acts as an endogenous dominant negative inhibitor of US3 function (Shin, 2006). The truncated US3 isoform may modulate US3 function across different cell types or different stages of infection, though the role of truncated US3 isoforms during infection awaits further characterization.

Perhaps US3 evolved dual mechanisms for class I retention to cope with the extensive heavy chain sequence and structural polymorphism present in a given outbred human population. Though no other human herpesviruses are known to inhibit ER chaperones, the murine herpesvirus γ-68 K3 gene product and adenovirus E19 likewise exploit tapasin to interfere with class I molecule assembly (Bennett *et al.*, 1999; Lybarger *et al.*, 2003).

Destruction of class I molecules via proteasomal proteolysis

Herpesviruses appear to have studied cellular pathways that control protein turnover and craftily manipulate multiple such pathways to reduce the abundance of the class I MHC molecule on the cell surface. One such pathway, utilized by HCMV and murine γ-herpesvirus-68, requires proteasomal proteolysis itself to break down newly synthesized class II molecules.

During the early period of HCMV infection, the ER-resident US2 and US11 gene-products are expressed with β, or delayed early kinetics. US2 and US11 independently bind to class I molecules undergoing assembly and rapidly target their ER dislocation, the rapid transfer from the ER to the cytosol. Once in the cytosol, class I heavy chains

are deglycosylated by a cellular N-glycanase and then degraded. Via emerging pathways that resemble cellular quality control pathways, US2 and US11 trick the cell into recognizing nascent class I molecules as misfolded proteins and selectively accelerate the rate constant of the dislocation reaction (Wiertz *et al.*, 1996a,b).

US2 is a 199-residue type I integral membrane protein, with an amino-terminal ER-lumenal domain comprised of an Ig-like fold, a single transmembrane domain, and a short carboxyterminal cytoplasmic tail (Gewurz *et al.*, 2001a). Although it targets a population of newly synthesized ER-resident class all I molecules for degradation, US2 recognizes a class I surface that apparently arises late in the biosynthesis and assembly of class I molecules in the ER (Gewurz *et al.*, 2001a). SiRNA-mediated knockdown of β2-microglobulin light chain expression blocks US2 association with and the dislocation of class I MHC molecules (Blom *et al.*, 2004). Recombinant US2 likewise associates with the folded class I, and not with the free class I heavy chains in vitro (Gewurz *et al.*, 2001b). Interactions between the US2 and class I ER-lumenal domains are required for subsequent removal of class I molecules from the ER: mutation of a single class I residue at the US2-binding site abrogates dislocation (Gewurz *et al.*, 2001a).

US2 associates with the class I MHC heavy chain at the junction between the peptide-binding cleft and the α3 domain. The class I binding surface recognized by US2 has several important properties. US2 chooses a relatively conserved surface on the highly polymorphic class I molecule, allowing it to interact with many class I alleles (Gewurz *et al.*, 2001a, b). The sequence of class I peptide cargo does not influence US2 binding, and US2 likely also binds with empty class I molecules awaiting receipt of peptide, as would be the case when US2 and US6 are coexpressed (Gewurz *et al.*, 2001a, b). Further, the proposed binding surface for the peptide-loading complex maps to a face of the class I MHC molecule opposite that of the US2 binding site. Thus, US2 should have access to class I even when associated with the multisubunit PLC, and so diminishes the probability of class I molecule escape from the ER.

NK-cells scan for the loss of class I surface expression, providing an important back-up function, as cells without class I MHC are no longer recognized by CD8+ T-cells. Upon recognition of cells that have lost surface expression of class I molecules, in particular the HLA-C, -E and -G locus products, NK cytotoxic activity is stimulated (Lanier, 2003). In contrast, NK cell inhibitory receptors specific for these class I loci restrain NK-cell activity against host cells with surface disposed class I MHC molecules. Interestingly, the US2 ER-lumenal domain dictates binding to class I molecules in a locus-specific fashion (Gewurz *et al.*, 2001b). US2, and perhaps by a similar mechanism US11, downregulates HLA-A and HLA-B, but not HLA-C, HLA-E molecules (Schust *et al.*, 1998; Furman *et al.*, 2000; Barel *et al.*, 2003). Such locus specificity may allow HCMV to selectively diminish antigen presentation to CD8+ T-cells, which rely heavily on HLA-A and HLA-B products, whereas NK-cell inhibitory receptors predominantly recognize HLA-C, HLA-E and HLA-G molecules. Further highlighting this dichotomy, the signal peptide of HCMV UL40 supplies a peptide ligand for HLA-E in a TAP-independent fashion, assuring that loaded HLA-E complexes can travel to the cell surface even during the expression of US2, US6 or US11 (Tomasec *et al.*, 2000).

US2 association does not significantly alter the conformation of the class I molecule (Gewurz, *et al.*, 2001a). How then does US2 initiate the dislocation of class I molecules from the ER? Though important molecular details of the US2 pathway remain to be elucidated, it appears likely that US2 recruits cellular factors following association with class I MHC. The US2 Ig-like fold has multiple additional binding surfaces that could associate with additional ER-resident proteins, for example the loops between its beta-strands that by analogy mediate immunoglobulin and TCR binding to antigens (CDR loops) (Gewurz *et al.*, 2001a). Alternatively, the US2 cytoplasmic tail is required for dislocation and may also interact with cytosolic or membrane proteins (Furman *et al.*, 2002). The intramembrane-cleaving protease, signal peptide peptidase associates with US2 and is required for dislocation of the class I MHC complex from the ER (Loureiro, 2006).

US2 triggers the mono-, di- and triubiquitination of folded molecules (Furman *et al.*, 2003). However, removal of all lysine residues from the class I cytoplasmic tail does not prevent dislocation (ubiquitination is not thought to occur in the oxidizing environment of the ER) (Furman *et al.*, 2003). A ubiquitin-independent step must therefore initiate dislocation, with ubiquitin attachment occurring either on the class I cytoplasmic tail, or on an ER-lumenal lysine residue following dislocation. Alternatively, ubiquitination of cellular machinery involved in assembly of the dislocation complex may be required to initiate class I MHC removal from the ER. Recombinant class I MHC molecules that lack lysine residues continue to be dislocated by US11, but not US2, even though CMV immuno-evasins require the activity of cellular E1 ubiquitin activating enzymes for US2-mediated dislocation (Hassink, 2006). Once in the cytosol, class I molecules are deglycosylated by cellular enzyme peptide: *N*-glycanase (PNGase or PNG1) (Blom *et al.*, 2004). *N*-glycanase cleaves the β-aspartyl-glucosamine bond of glycans attached to Asn residues. The products of the *N*-glycanase reaction

are a free oligosaccharide and a peptide containing an Asp residue at the site of hydrolysis (formerly an Asn). The class I heavy chain is then rapidly degraded by the proteasome. Deglycosylation precedes proteasomal degradation, as siRNA-mediated knock-down of PNGase expression in US2+ cells results in the accumulation of glycosylated class I heavy chains in the cytosol (Blom *et al.*, 2004).

US2 binds to the folded class I complex with micromolar affinity, and the recombinant US2-HLA-A2 complex is sufficiently stable to survive consecutive size exclusion and ion exchange chromatography steps. The stability of the complex raises the question of whether it must be disassembled prior to exit from the ER (Gewurz *et al.*, 2001a, b). If so, dissociation would require ER machinery that has yet to be identified. Rather, experimental evidence increasingly suggests that US2 dislocates intact class I MHC molecules. Chimeric class I MHC molecules bearing an ER-lumenal green fluorescence protein (GFP) undergo US2- and US11-mediated ER dislocation (as evidenced by the Asn-to-Asp sequence conversion that accompanies PNGase-mediated removal of the N-linked glycan, previously attached in the ER) (Fiebiger *et al.*, 2002). Fluorescent, deglycosylated intermediates can be recovered from the cytosol and imply that the GFP moiety traversed the membrane in a folded state. Likewise, chimeric class I MHC molecules with an appended ER-lumenal dihydrofolate reductase (DHFR) domain, a tightly folded moiety of considerable size, undergo dislocation by US2 and US11. Again, deglycosylated class I heavy chains with a folded DHFR can be retrieved from the cytosol (Tirosh *et al.*, 2003).

Which ER membrane pore might accommodate a complex of folded molecules? Preliminary experiments implicated the Sec61 pore in ER dislocation, a membrane complex that allows insertion of secretory and membrane proteins into the ER during their biosynthesis (Wiertz *et al.*, 1996a,b). However, it has been difficult to identify a complex between Sec61 and proteins undergoing dislocation. Importantly, the glycosylated US2–class I complex would not likely be accommodated by the Sec61 translocon, given the strict upper limit of the pore diameter suggested by the structure of the analogous archea SecY complex. Physiological measurements of the Sec61 translocon itself also suggest that a folded US2-class I complex would not be allowed through (Hamman *et al.*, 1997; Van den Berg *et al.*, 2004). By analogy with the US11 pathway (see below), an unidentified pore most likely accommodates the US2–class II MHC complex.

US11 is a 215-residue ER-resident glycoprotein that is homologous with US2. US11 likewise directs the degradation of class I MHC heavy chains within minutes of their synthesis (Wiertz *et al.*, 1996a,b). US11 induces the expression of X-box binding protein 1 (XBP-1), a key transcription factor that regulates the unfolded protein response (UPR) pathway. XBP-1 expression coincides with that of US11 in human cytomegalovirus infected foreskin fibroblasts, and the UPR appears to facilitate US11-medicated degradation of class I MHC (Tirosh, 2005). In the presence of US11, polyubiquitinated conjugates are affixed to class II heavy chains, in a fashion that appears to be independent of their tertiary structure. Whereas US2 causes addition of 1–3 ubiquitin moieties only to folded class II molecules, US11 attacks unfolded class I heavy chains and folded class I molecules alike. Ubiquitination is required for the US11 ER dislocation reaction (Kikkert *et al.*, 2001; Shamu *et al.*, 2001; Furman *et al.*, 2003). Cells that express both US11 and a temperature-sensitive mutant of the E1 ubiquitin-activating enzyme demonstrate normal US11-mediated degradation of class I molecules at permissive temperatures. However, upon shift to non-permissive temperatures, the class I heavy chains remain within the ER (Kikkert *et al.*, 2001). Isopeptide linkage between the ε-amino group of target proteins and ubiquitin residue 48 slates multiple proteins for proteasomal destruction, and likewise appears required for extraction of the class I heavy chain from the ER membrane in the US11 pathway (Flierman *et al.*, 2003; Varadan *et al.*, 2004). US11 may direct the ubiquitin-conjugating enzyme E2-25K, perhaps along with the E3 ligases MARCHVII/axotrophin or gp78, to attach polyubiquitin conjugates to class I MHC (Flierman, 2006).

Whereas the US11 cytoplasmic tail is dispensable for its function, the US11 transmembrane domain plays an active role in dislocation (Furman *et al.*, 2002; Lilley *et al.*, 2003). US11 Gln-192 is required for interactions with host proteins within the plane of the membrane, in particular Derlin-1 (Lilley and Ploegh, 2004). Derlin-1 is a recently identified homologue of yeast Der1p, an ER membrane protein that is required for the degradation of a subset of misfolded yeast ER proteins (Knop *et al.*, 1996). A Derlin-1 dominant-negative mutant prevents US11-, but not US2-mediated class I dislocation (Lilley and Ploegh, 2004). The integral membrane protein VIMP associates with Derlin-1 and recruits a complex of cytosolic proteins, the cytosolic p97 ATPase (also known as VCP, or CDC48 in yeast) and its cofactor complex Ufd1-Npl4 (Ye *et al.*, 2001, 2004). A complex containing US11, ubiquitinated class I heavy chain, Derlin-1, VIMP, and p97 has been detected (Ye *et al.*, 2004; Lilley, 2005). Further, p97 activity is required for US11-mediated heavy chain retro-translocation (Ye *et al.*, 2001, 2004). The P97–Ufd1–Npl4 complex recognizes proteins undergoing ER retro-translocation and provides the driving force for protein movement into the cytosol (Ye *et al.*, 2001). Thus, P97 captures MHC heavy chains marked by US11 for

disposal, and following poly-ubiquitination, extracts them from the ER membrane. The multiple membrane spanning Derlin-1 interacts with class I heavy chains both before and immediately after dislocation to the cytosol. Perhaps in concert with accessory proteins, Derlin-1 may comprise the exit channel utilized by the US11 pathway (Lilley and Ploegh, 2004; Ye *et al.*, 2004). A dominant negative Derlin-1 mutant blocks US11-, but not US2-dependent degradation of class I molecules (Lilley and Ploegh, 2004).

The apparent redundancy of US2 and US11 appears to contradict the general parsimony of viral genomes brought about by selective pressure. Yet, US2 and US11 are present in the genomes of multiple clinical HCMV isolates (Erica Mayer, Rebecca Tirabassi and Hidde Ploegh, data unpublished). Retention of both US2 and US11 may benefit CMV in several fashions. The two immuno-evasins utilize distinct dislocation pathways, and perhaps maximize the number of complexes that can be extruded during a given time. Since US2 and US11 recognize distinct intermediates in class I assembly, perhaps US2 serves an editing function and catches proteins missed by US11 (it is known whether for example US11 can bind to class I molecules occupied by PLC). Alternatively, either pathway may be preferred in a given cell type, a notion that is supported by experimental data from HCMV infection of human dendritic cells and from analogous MCMV inhibitors of class I (Hengel *et al.*, 2000; Rehm *et al.*, 2002). Finally, the distinct US2 and US11 binding sites on class I allow US2 and US11 to target distinct subsets of class I alleles and thereby counter class I polymorphism (Machold *et al.*, 1997; Gewurz *et al.*, 2001a,b; Barel *et al.*, 2003). The MCMV immuno-evasins likewise bind class I molecules in an allele-specific fashion and cooperatively prevent antigen presentation to a variety of CTL clones (Kavanagh *et al.*, 2001a,b).

The murine γ-herpesvirus-68 evolved a distinct pathway for proteasomal proteolysis of ER-resident class I molecules. The MK3 locus encodes an E3 ubiquitin ligase that directs the ubiquitination of the class I MHC cytosolic tail. MK3 forms a multiprotein complex with class I molecules, tapasin and TAP, and targets properly folded class II molecules during the peptide loading stage (Lybarger *et al.*, 2003). Association with tapasin/TAP appears to determine the class I allele-specificity (Wang *et al.*, 2004). Although initially characterized as a member of the plant homeodomain/leukemia-associated protein (PHD/LAP) family, a recent solution structure of the MK3 cytosolic domain reveals it to be a variant member of the RING domain family (Dodd *et al.*, 2004). RING motifs are 8 kDa zinc-binding domains that serve as adaptors for E2 ligases. RING domains do not generally possess their own enzymatic function, but instead provide substrate selectivity by directing E2 ubiquitin ligase activity (Freemont, 2000). The MK3 ring domain associates with cellular ubiquitin-conjugating enzymes UBCH5A-C and UBCH13, which have previously been shown to function in concert with cellular RING domain proteins. MK3 ubiquitination of the class I MHC molecule stimulates its removal from the ER (Bartee *et al.*, 2004). Akin to US11, MK3 requires the cellular proteins Derlin-1 and AAA-ATPase p97 for dislocation (Wang, 2006).

Disruption of the class I secretory pathway

Following receipt of peptide cargo, stable class II complexes exit the ER and traverse the Golgi *en route* to the cell surface. Herpesviruses once again a specific step in class I molecule biosynthesis, interfering with export of class I molecules. The HCMV US10 gene-product encodes a type I integral membrane glycoprotein that associates with class I molecules and delays egress of the class I MHC molecules from the ER (Furman *et al.*, 2002). The mechanism of US10 action remains uncertain. While US10 expression does not appear to alter the kinetics of class II MHC molecule degradation by US2 or US11, perhaps US10 serves to retard class I molecule egress from the ER and thereby minimize the probability that class I molecules escape US2/11 mediated degradation. For instance, at the onset of the early period of viral infection, US2 and US11 likely encounter a large population of class I molecules retained by US3 during the IE period.

HCMV US8 encodes a 26-kDa type I membrane glycoprotein that partially co-localizes with markers of the endolysosomal pathway. Although US8 associates with free class I MHC heavy chains within the ER, the significance of US8 association has not been fully elucidated: US8 expression does not appear to alter the maturation of class I products in cellular transfectants (Tirabassi and Ploegh, 2002). It remains possible that US8 exerts its effect on class II trafficking in concert with other CMV immuno-evasins. Indeed, such cooperative activity has been observed with the MCMV class II inhibitors m4 and m152 (Kavanagh *et al.*, 2001a,b). The MCMV m152 gene-product (gp40) blocks the export of class I from an ER-Golgi intermediate compartment (ERGIC) (Ziegler *et al.*, 2000). Although the mechanism remains to be fully defined, m152-mediated class I molecule retention does not require the m152 cytoplasmic tail or transmembrane domains. Further, class I molecules continue to be retained in the secretory pathway after the complex dissociates (Ziegler *et al.*, 2000). The m4 gene-product forms biochemically distinct complexes with differential stabilities with class I molecules in the presence and absence of m152 (Kavanagh *et al.*, 2001a,b).

Cell surface expression of class I molecules is reduced upon VZV infection, an effect that has been observed in both human T-cells in the SCID-hu thymus/liver mouse model and in skin biopsy specimens (Abendroth *et al.*, 2001a,b; Nikkels *et al.*, 2004). Microscopic and biochemical experiments demonstrate the accumulation of class I MHC molecules in the Golgi compartment in VZV-infected cells (Abendroth *et al.*, 2001a,b). The ORF66 gene-product is expressed during the early period of virus infection and serves to retain class I molecules in the Golgi complex. Cellular transfectants that express ORF66 demonstrate reduced cell surface expression of class I MHC (Abendroth *et al.*, 2001a,b). The molecular mechanism by which VZV halts class I in the Golgi has yet to be fully elucidated. The UL49.5 gene-product of several varicelloviruses including bovine herpesvirus I, pseudorabies virus, and equine herpesvirus 1, though not of varicella zoster, blocks the TAP peptide transporter through conformational arrest and subsequent proteasomal degradation (Koopers-Lalic, 2005).

Rerouting of class I molecules from the Golgi to the endolysosomal pathway

The human herpesvirus 7 (HHV-7) interferes with the class I MHC antigen presentation via the U21 gene product. U21 is a 55-kDa integral membrane glycoprotein that associates tightly with folded class I molecules within the ER, shortly after class I molecule biosynthesis (Hudson *et al.*, 2001). Although the molecular affinity remains unknown, U21 association with multiple class I alleles appears strong and survives treatment with .01% SDS in cellular extracts (Hudson *et al.*, 2001). The complex travels at least as far as the trans-Golgi network, where U21 diverts class I to the endolysosomal pathway. class I molecules are then degraded by lysosomal proteases.

MCMV employs a similar strategy via its m6 gene product. M6 is a type I ER-resident integral membrane glycoprotein that likewise associates with class I molecules within the ER and redirects them for lysosomal destruction. M6 utilizes a dileucine sorting signal within its cytoplasmic tail to achieve relocalization to lysosomes (Reusch *et al.*, 1999). Surprisingly, although the U21 cytoplasmic tail can likewise utilize a dileucine-like sorting motif to mediate its own intracellular sequestration, U21's cytoplasmic domain is dispensable for its function. U21 truncation mutants that lack cytoplasmic tails continue to target class I MHC molecules to lysosomes (Hudson *et al.*, 2003). Perhaps the U21 cytoplasmic tail is required for other U21 functions, for instance retrieval of surface-disposed class I molecules?

Akin to most herpesvirus immuno-evasins of the class I pathway, U21 demonstrates no significant homology with cellular proteins. Even HHV-6 U21, with its 50% amino acid similarity to HHV-7 U21, does not appear to interact with class I MHC molecules (Hudson *et al.*, 2001). The function of HHV-6 U21 remains to be determined.

Retrieval of cell surface class I molecules

HHV-8 relies upon two type III integral membrane proteins to perturb the class I MHC pathway during the early lytic cycle of viral replication, K3 and K5 (also called MIR-1 and MIR-2, for modulator of immune recognition) (Coscoy and Ganem, 2003). K3 and K5 function independently and utilize interactions within the lipid bilayer to recognize their targets, highlighting the skilled manipulation of cell biology co-opted by herpesviruses to subvert class I (Ishido *et al.*, 2000; Sanchez *et al.*, 2002). Additional cellular cofactors may be involved K3/5 recognition of class I MHC.

K3 and K5 share approximately 40% amino acid identity and are predicted to have cytoplasmic amino- and carboxy-terminal domains, two transmembrane domains, and a short ER lumenal domain (Coscoy and Ganem, 2003). Though initially characterized as a PHD/LAP domain, it appears likely that K3/K5 instead contain a variant RING domain (Scheel and Hofmann, 2003; Bartee *et al.*, 2004; Dodd *et al.*, 2004). Surprisingly, although they are homologous to the γ-herpesvirus 68 MK3 gene product that targets class I for ER dislocation, K3/5 instead re-route mature class I molecules to the endolysosomal pathway. Inhibition of lysosomal acidification by chloroquin or bafilomycin prevents class II molecule degradation (Fruh *et al.*, 2002). Covalent attachment of short-ubiquitin chains can mediate multiple intracellular trafficking processes, such as endocytosis and sorting to multi-vesicular bodies (MVB). Thus, K3 and K5 appear to function as E3 ligases and thereby initiate internalization of cell surface-disposed (or perhaps recycling) class I molecules to MVBs (Coscoy and Ganem, 2003). The K5 RING domain promotes ubiquitin transfer in an ATP- and E2-dependent reaction in vitro (Coscoy *et al.*, 2001). Disruption of the K3 RING domain prevents class I molecule internalization, even though association with class I still takes place (Hewitt *et al.*, 2002).

The majority of K3 and K5 molecules localize to the ER. Nonetheless, class II molecules exit the ER apparently unscathed and reach the plasma membrane with normal kinetics in the presence of K3 and K5 (Coscoy and Ganem, 2003). Perhaps ubiquitination of class I cytoplasmic tails might occur while the molecules are still in the ER and thereby mark them for eventual plasma membrane internalization. Alternatively, the minority of K3 and K5 that travels along the secretory pathway may be the active population. The available experimental evidence favors the latter model (Hewitt *et al.*, 2002; Coscoy and Ganem, 2003).

Ubiquitination of the conserved class I MHC cytoplasmic tail Lys-340 residue provides an internalization signal (Hewitt *et al.*, 2002). Interestingly, in an apparently novel enzymatic reaction, K3 can catalyze ubiquitination of cysteine residues via thiolester bond formation. class I MHC molecules that lack lysine residues in their cytoplasmic tails undergo cysteine-ubiquitination, which is sufficient to target class I for endocytosis and degradation (Cadwell, 2005). Sorting of ubiquitinated surface membrane proteins to MVB requires the activity of multiple cellular proteins, including dynamin and tumor susceptibility gene-101 (TSG101) (Hewitt *et al.*, 2002; Coscoy and Ganem, 2003). SiRNA-mediated knockdown of TSG101 expression prevents K3- and K5-mediated endocytosis of class I MHC molecules.

K3 subsequently directs the internalized class I molecules to lysosomes, perhaps via the concerted action of two distinct sorting motifs: a YXXϕ motif (where Y represents tyrosine; X signifies any residue; ϕ signifies a hydrophobic residue) and a diacidic cluster region. YXXϕ motifs are recognized by the adaptor protein (AP) complexes AP-1, AP-2 and AP-3, and direct trafficking into clathrin coated vesicles (Owen and Evans, 1998). The K3 Y_{152}AAV motif directs class I molecules to the *trans*-Golgi network via AP-1. The K3 diacidic cluster motif subsequently targets class I to the lysosomal compartment (Means *et al.*, 2002). Acid cluster dileucine motifs sort proteins from the trans-Golgi network to lysosomes via cargo proteins, including the so called Golgi-localized, γ-ear-containing, ARF-binding proteins (GGAs) (Takatsu *et al.*, 2001).

Proteasome activity also plays a key role in K3-mediated sorting of class II to the endocytic compartment, and small molecule proteasome inhibitors prevent K3-mediated delivery of class I MHC molecules to the dense endosomal compartment (Lorenzo *et al.*, 2002). Proteasome inhibition does not appear to increase cell surface expression of class I, however. Thus, the specific mechanistic role of the proteasome in the K3 pathway remains to be determined. Once within the endosomal compartment, cysteine protease activity removes the class I cytoplasmic tail, and an aspartyl protease subsequently cleaves class I within the plane of the membrane, liberating a soluble class I MHC molecule (Lorenzo *et al.*, 2002).

Poxviruses and several other herpesviruses also encode an immuno-evasin of the K3 family that likewise target class I molecules, suggesting a common evolutionary origin. These unrelated viruses appear to have acquired K3 and K5 from the mammalian MARCH family, a group of 9 subcellular integral membrane proteins that target glycoproteins for lysosomal destruction (Bartee *et al.*, 2004). Like K3 and K5, MARCH IV and MARCH IX possess ubiquitin-ligase activity against the class I cytoplasmic tail and stimulate endocytosis (Bartee *et al.*, 2004). MARCH proteins may participate in the regulation of endogenous class I surface expression, perhaps in a fashion analogous to regulation of growth factor receptors endocytosis by E3 ligases (Hicke, 2001). Interestingly, the extreme constraints on the retroviral genome may have forced HIV-1 to convergently evolve a unique approach for class I MHC inhibition via its Nef gene-product, which has no homology to MARCH proteins. Nef is a cytosolic protein that appears to promote endocytosis of class I molecules by a distinct pathway that involves cellular phosphofurin acidic cluster sorting protein-1 (PACS-1) (Piguet *et al.*, 2000).

K3 and K5 are adjacent in the HHV-8 genome and likely arose via gene duplication. Once again, why does a herpesvirus retain homologous factors with apparently overlapping function and kinetics? As with HCMV, K3 and K5 may counteract allelic variation (albeit less prevalent among class I cytoplasmic tails) and may function to varying degrees among various cell types. Indeed, K5 significantly downmodulates HLA-A and HLA-B locus products, whereas K3 downregulates HLA-A, -B, -C and -E molecules (Ishido *et al.*, 2000). Further, K5 targets additional proteins for endocytosis, and additional K3 targets may likewise exist. Studies of the MARCH family reveal an analogous an functional division: several MARCH proteins have a spectrum similar to K3, while a second subset more closely resemble K5 target specificity (Bartee, *et al.*, 2004). Indeed, the RING domain of K3 can be replaced with the corresponding domain of a cellular MARCH homologue without altering K3 function (Goto *et al.*, 2003).

Interference with class I molecules at the cell surface

The cell surface itself represents the final compartment for the inhibition of class I MHC display of antigenic peptides. Though no examples of physical interference with TCR recognition of class I/peptide complex are yet known among the human herpesviruses, MCMV m4 appears to do so. The m4 gene-product encodes a 34-kDa type I integral membrane glycoprotein (gp34) that initially associates with folded class II molecules within the ER. The m4-class II complex is then exported to the cell surface. Where M4 may sterically hinder TCR association the class I MHC–peptide complex. m4 thereby blocks antigen presentation at the cell surface to $K^{(b)}$-restricted clones (Kavanagh *et al.*, 2001a,b). Cell tropism has been observed for m4 function, with greater inhibitory function present in macrophages than in fibroblasts (LoPiccolo *et al.*, 2003), and macrophage infection with MCMV deletion mutants suggest synergistic function between its three class I immuno-evasins (LoPiccolo *et al.*, 2003).

Interference with the immunological synapse

T-cells form stable associations with antigen presenting cells (APCs) known as the immunological synapse to facilitate cell signaling. Intracellular adhesion molecule-1 (ICAM-1, or CD54) is a member of the Ig-superfamily and plays a key role in stable synapse formation, binding tightly to its T cell counter-receptor, LFA-1. ICAM-1 represents another cellular target of K5 and, like class I, is re-routed for lysosomal degradation, apparently by the same pathway (Ishido *et al.*, 2000; Coscoy *et al.*, 2001). Reduction of ICAM-1 cell surface expression has been observed on infected keratinocytes from skin biopsy specimen of VZV-infected patients (Nikkels *et al.*, 2004). HHV-8 infection also leads to the downregulation of PE-CAM (CD31) surface expression, and may even occur during the latent stage of infection (Tomescu *et al.*, 2003).

Interference with costimulation

Interactions between numerous cell surface receptors tightly regulate the context of T-cell activation to restrain the production of T-cell toxins. Along with recognition of an MHC–peptide complex, initial T-cell activation requires simultaneous, costimulatory signals from the APC. Interactions between costimulatory molecules and their counter-receptors present an additional step in T-cell activation that herpesviruses disrupt.

Professional APCs express the costimulatory molecules B7-1 and B7-2, which independently can provide an important second signal for T-cell activation. HHV-8 K5 and murine-γ68 MK3 perturb this control point of adaptive immunity. Both K5 and MK3 target B7-2 molecules for degradation, apparently by the same pathways utilized for class I degradation (Ishido *et al.*, 2000; Coscoy *et al.*, 2001; Wang *et al.*, 2004). Although B7-1 and B7-2 are both functionally and structurally similar, B7-1 lacks cytoplasmic tail lysines and escapes both K5 and MK3. Introduction of lysines into the B7-1 cytoplasmic tail confers susceptibility to K5-mediated degradation (Coscoy *et al.*, 2001). Why should two herpesviruses destroy B7-2 and not B7-1? Perhaps such selectivity helps the virus to strike a balance with the host. For example, mice deficient in both B7 molecules have a more profound immunodeficiency than those lacking B7-2 alone (Borriello *et al.*, 1997). Inhibition of just B7-2 may enable the establishment of persistent infection but limit damage to its host. Indeed, MCMV encodes a class I-like molecule that likewise may function to reduce host damage. The m157 gene-product encodes a class I-like NK-cell ligand that allows control of MCMV acute infection. Strains whose NK cells fail to recognize this CMV-encoded ligand do not survive acute infection, whereas persistent infection is established only in strains whose NK cells control acute viremia (Arase *et al.*, 2002; Bubic *et al.*, 2004).

Though at first glance downmodulation of B7-2 appears unique to gamma-herpesviruses, the targeting of B7 molecules was most likely usurped from the mammalian genome. Several MARCH proteins, including MARCH I, MARCH II and MARCH VIII (c-MIR) likewise target B7-2, apparently by the same pathway (Goto *et al.*, 2003; Bartee *et al.*, 2004). Further, a subset of MARCH proteins (IV and IX) target class II, whereas others (I and VIII) target both B7-2 and class II, reminiscent of K3 and K5, respectively (Bartee *et al.*, 2004). The K5 gene-product targets PE-CAM for lysosomal degradation, apparently via the same pathway described previously for disposal of class I MHC molecules by K5 (Mansouri, 2006).

Interference with APC function

Dendritic cells (DCs) are professional antigen-presenting cells that play a crucial role in stimulation of adaptive T-cell responses. Multiple members of the human herpesvirus family decrease the capacity of DC to stimulate T-cells.

HCMV infects a subset of DCs and disrupts their activity. HCMV-infected DCs demonstrate reduced capability to stimulate T cell proliferation and cytotoxicity, a phenotype that has been attributed to pleiotropic effects on the DC. These include reduced surface expression of multiple proteins (including class I MHC and II, B7-1 and B7-2 and CD40) as well as reduced capacity to secrete stimulatory cytokines and a functional unresponsiveness to maturation stimuli (Hertel *et al.*, 2003). Monocyte-derived DCs infected with HCMV do not migrate toward several lymphoid chemokines, potentially delaying the kinetics of primary CD8+ T-cell activation (Moutaftsi *et al.*, 2004). Functional paralysis of multiple DC functions has likewise been observed following MCMV infection of DCs (Andrews *et al.*, 2001). Further, VZV infection of mature dendritic cells causes downregulation of a similar subset of cell surface molecules (Morrow *et al.*, 2003). Infection of DCs can even facilitate VZV dissemination and support infection of T-cells themselves (Abendroth *et al.*, 2001). VZV-infected T-lymphocytes play an important role in supporting persistent viremia.

HSV-1 can infect immature DCs and inhibit their maturation (Kobelt *et al.*, 2003). EBV infection of monocytes can similarly prevent their development into dendritic cells, an activity that is observed even with UV-inactivated virion. The trimolecular gp25-gp42-gp85 complex appears to exert

this inhibitory activity via interactions with class II MHC at the monocyte membrane (Li *et al.*, 2002). EBV-infected monocytes also demonstrate significantly reduced phagocytic activity, though the mechanism remains incompletely understood (Savard *et al.*, 2000).

HSV also interferes with DC function by perturbing CD83, a 45-kD cell surface adhesion molecule important for DC interaction with T-cells. Within hours after HSV infection, CD83 cell surface expression is significantly reduced, a phenotype that has been attributed to re-routing of CD83 molecules to the endolysosomal pathway. The molecular mechanism, including the responsible HSV factor(s), remains to be determined (Kruse *et al.*, 2000). However, a similar reduction in DC CD83 surface disposition during CMV infection has been attributed to TGF-β expression by HCMV-infected fibroblasts (Arrode *et al.*, 2002).

HSV-1 infection of B cells strongly inhibits their ability to stimulate CD4+ T-cells (Barcy and Corey, 2001). The immediate-early HSV US1 gene-product ICP22 is both necessary and sufficient for this phenotype, by an unknown mechanism.

Stunning of Cytotoxic T-cell activity

HSV-1 infection of target cells appears to exert an inhibitory effect *in trans* on CTLs. Inactivated, or stunned, CTL are transiently incapable of cytokine synthesis or cytotoxic granule release in response to TCR ligation (Sloan *et al.*, 2003). The phenomenon requires close cell–cell contact between CTL and the HSV-infected target cell (Sloan *et al.*, 2003), and HSV penetration into T-cells.

Though no *de novo* viral transcription or translation is required, the TCR signal transduction cascade is inhibited at the level of the linker for activation of T cells (LAT). Through an uncharacterized mechanism, HSV infection reduces LAT phosphoryation (Sloan, 2006).

CTL escape mutants

EBV, like other herpesviruses, is a genetically stable virus. Nonetheless, mutations occur at low frequency, and may allow EBV to adapt to populations where a particular class I MHC allele or haplotype prevails. For example, mutations that affect class I binding (anchor residues) are regularly detected in HLA-A11 epitopes of South East Asia EBV isolates, where greater than 50% of the population expresses HLA-A11. In contrast, HLA-A11 epitope loss is much less common in central African isolates, where HLA-A11 is absent (de Campos-Lima *et al.*, 1993).

Ligation of inhibitory receptors

HCMV and MCMV each encode a viral class I MHC molecule homologue. The HCMV UL18 molecule forms a complex with β2-microglobulin and peptide, and is exported to the cell surface. Interestingly, UL18 is resistant to the activity of the HCMV class I immuno-evasins (Park *et al.*, 2002). Rather than presenting antigenic peptide to CD8+ T-cells, UL18 binds tightly to LIR-1 (ILT2, or CD85), an inhibitory receptor expressed on monocytes, dendritic cells and lymphocytes (Willcox *et al.*, 2003). UL18 binds to LIR1 with >1000-fold higher affinity than the association between class I MHC and LIR1 (Willcox *et al.*, 2003). Though it is not yet clear to what extent UL18 modifies lymphocyte function, the widespread expression pattern of LIR-1 suggests that UL18 may potentially alter multiple steps in T-cell activation.

Inhibition of T-cell receptor signaling

Several herpesviruses alter the interaction between APC and T-cells from within T-lymphocytes. The lymphotropic HHV-6A has been reported to reduce expression of the TCR/CD3 complex on the transcriptional level, with transcriptional downregulation of multiple components of the T-cell receptor signaling apparatus. An HHV-6 factor expressed during the late stage of virus infection has been implicated (Lusso *et al.*,1991). HCMV UL20 encodes a protein with amino acid sequence similarity to both the constant and variable regions of the T-cell receptor γ chain (Beck and Barrell, 1991). Further studies are required to elucidate the role of the putative HCMV TCR γ homologue.

Though not yet observed among the lymphotropic human herpesviruses, the Herpesvirus saimiri (HVS) interferes with downstream components of the TCR signal transduction pathway. The HVS Tip (tyrosine kinase–interacting protein) gene-product interferes with early signaling events of the TCR signal transduction pathway (Cho *et al.*, 2004). Tip constitutively localizes to lipid rafts, where it binds to the SH3 domain of Lck, a member of the Src tyrosine kinase family. Tip interacts also with endosomal protein 80 and induces endosomal vesicle formation, thus sequestering Lck. Tip may also interfere with T-cell signaling by promoting the internalization of lipid rafts (Cho *et al.*, 2004b).

Erroneous T-cell activation

EBV infection transactivates the env gene, a superantigen encoded by the human endogenous retrovirus HERV-K18. LMP-2 activity is sufficient for env induction at the

transcriptional level (Sutkowski *et al.*, 2001). Env preferentially activates the TCR Vβ13 subset of CD4+ T-cells (Sutkowski *et al.*, 2001). While it remains to be determined if env transactivation supports EBV immuno-evasion or is incidental, it is noteworthy that superantigen activity can deregulate the establishment of specific immunity. In a more direct fashion, MCMV primes the development of CD8+ T-cells specific for virus epitopes that are not presented in relevant tissues, where they are hidden by immuno-evasin activity (Holtappels *et al.*, 2004).

Disruption of class II MHC antigen presentation

The class II MHC antigen presentation pathway presents antigenic peptides derived from the extracellular compartment to CD4+ T-cells. Whereas class II MHC molecules are expressed by most nucleated cells, class II expression is largely restricted to APC, such as DC, monocyte/macrophages, and B-cells, though immune cytokines can induce class II expression on a variety of other cell types. APC deliver pathogens (or material derived from pathogens) via phagocytosis or receptor-medicated endocytosis into the endolysosomal pathway for destruction (Denzin and Cresswell, 1995).

Class II MHC molecules are heterotrimeric complexes that contain the class II α and β chains, and an antigenic peptide. There are three class II loci: DP, DQ and DR. The class II αβ heterodimer undergoes folding within the ER and associates with an accessory protein called the invariant chain (Ii). Ii prevents class II peptide loading within the ER, and by virtue of a dileucine motif within its cytoplasmic tail, shuttles the nascent class II molecules through the TGN to the lysosomal compartments (Wubbolts and Neefjes, 1999). Ii is degraded within the lysosomal compartment, where the class II molecules encounter peptide-fragments produced by lysosomal proteolysis of internalized material. The MHC-encoded HLA-DM molecule then catalyzes exchange of the class II-bound Ii peptide called CLIP with higher affinity, lysosomally-derived peptide ligands. Peptide-loaded class II molecules are exported to the cell surface for antigen presentation to CD4+ T-cells. Not surprisingly, herpesviruses manipulate antigen presentation by the class II MHC pathway as well.

Interference with class II expression

The expression of class II MHC molecules is under the control of both constitutive and inducible elements (Boss 1997). Class II is constitutively expressed on APCs, activated T-cells, and thymic epithelial cells, whereas a subset of cells upregulate class II MHC expression in an IFN-γ dependent fashion. In response to IFN-γ, the Jak/STAT signaling pathway is activated and translates the IFN-γ signal into transcriptional responses. Stat1α activity upregulates expression of the class II transcriptional activator CIITA, which in turn drives the expression of class II molecules (Boss, 1997). Fibroblasts, endothelial and epithelial cells all upregulate CIITA in response to IFN-γ (Boss, 1997).

HCMV interferes with the IFN-γ pathway of class II upregulation by several distinct mechanisms. During the immediate-early and early phases of virus infection, an HCMV factor targets JAK1 to the proteasome (Miller *et al.*, 1998). MCMV also interferes with IFN-γ upregulation of class II transcription, though apparently does not involve STAT1α perturbation (Heise *et al.*, 1998). Also, as mentioned earlier, MCMV M27 targets STAT2 for protosomal degradation. VZV also interferes with Jak/STAT signaling, apparently by the inhibition of Jak/STAT2 protein synthesis (Abendroth and Arvin, 2001).

Manipulation of class II molecules

The HLA-DR-α and HLA-DM-α molecules are targeted for proteasomal degradation in cells that are engineered to overexpress HCMV US2 (Tomazin, *et al.*, 1999). US3 has also been shown to interact with class II molecules and to retain them in the ER (Chevalier and Johnson, 2003). The observation that HLA-DRα and HLA-DMα are targeted for degradation by US2 is not readily explained by the structural data from the US2-HLA-A2 complex, as the class I binding surface recognized by US2 is not conserved on class II molecules (Gewurz *et al.*, 2001). Thus, it appears likely that a different US2 binding site is required for the downmodulation of class II MHC molecules. Likewise, though tapasin plays a key role in US3 interactions with class I molecules, tapasin is not known to interact with class II MHC complexes. Perhaps US3 might interact with the invariant chain in an analogous fashion in its class II pathway? Alternatively, US3 may also use a different mechanism altogether to retain class II molecules in the ER. In any case, it will be interesting to determine whether US2 and US3 alter the class II pathway in HCMV-infected cells.

HCMV has also been reported to alter trafficking of class II molecules within infected cells, causing the retention of vesicles bearing class II molecules in an aberrant perinuclear distribution (Cebulla *et al.*, 2002). A similar effect of HCMV has been observed in latently infected cells, where a block in protein trafficking causes the accumulation of class II molecules in intracellular vesicles. The responsible HCMV factor(s) have not been identified, but do not involve the US2–11 gene products (Slobedman *et al.*, 2002). Murine infection models demonstrate HSV-mediated

intracellular redistribution of class II MHC molecules within the CNS (Lewandowski *et al.*, 1993). Further studies are required to better define the biology underlying this observation.

The EBV BZLF2 gene-product (gp42) is a 42-kDA type II integral membrane glycoprotein that binds to class II MHC molecules. BZLF2 has been reported to interfere with CD4+ T-cell activation (Spriggs *et al.*, 1996). Gp42 is known to associate with class II MHC molecules to facilitate EBV entry into B cells. The molecular structure of the gp42–class II MHC complex has been determined, and gp42 belongs to the C-type lectin family and has homology with a family of human NK-cell receptors that bind to class II MHC (Mullen *et al.*, 2002).

gp42 occurs in two forms, including a soluble form generated by proteolytic cleavage within the ER. Soluble gp42 is secreted, and may inhibit cell surface class II MHC recognition by CD4+ T-cells. Secreted gp42 was detected during EBV lytic replication (Ressing, 2005).

Conclusions

Selective pressure exerted by adaptive immunity has led herpesviruses to convergently evolve a surprising number of immuno-evasins that specifically dysregulate T-cell responses. Even so, numerous herpesvirus gene-products have yet to be ascribed functions, in particular factors required for growth in vivo but dispensable for growth in cell culture. Further mechanisms of T-cell evasion by herpesviruses are sure to be discovered. Multiple herpesvirus-encoded micro-RNAs have been discovered, and may well serve as immunoevasins, as has been described for other viruses (Sullivan, 2005). With the set of immuno-evasins better defined, an ongoing challenge will be to define the specific role of T-cell immuno-evasins during the various stages of the herpesvirus lifecycle. An enhanced understanding of how herpesviruses solve the immunological puzzles faced during primary infection, dissemination, establishment of latency, reactivation, and sporadic shedding will be a challenging, though important endeavor. Such knowledge should directly lead to the development of novel antiviral agents, vaccines, gene therapy, and permit the application of immuno-evasins themselves to immunological disease states outside of viral biology.

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63

Subversion of innate and adaptive immunity: immune evasion from antibody and complement

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Many herpesviruses encode immune evasion molecules that interfere with activities mediated by antibody and complement, suggesting the importance of antibody and complement in host defense against herpes infections. How does this observation reconcile with the clinical findings that severe infections develop mostly in subjects with T-cell deficiencies, such as transplant recipients or those with advanced HIV infection? An explanation that we favor is that T-cells assume a pivotal role in host defense partly because herpesviruses are very effective at limiting the activities of antibody and complement. Support for this hypothesis comes from experimental studies using mutant HSV-1 strains defective in antibody and complement immune evasion that demonstrate a marked increased in effectiveness of antibody and complement in host defense against the mutant viruses (Lubinski *et al.*, 2002).

Newborns lack mature T-cell repertoires and generally have low serum complement levels; therefore, observations in human newborns provide opportunities to assess the contributions of antibodies independent of T-cells and perhaps complement in host defense against herpesviruses. The severity of HSV and CMV infection in the fetus and newborn are greatly reduced when the infection in the mother is recurrent rather than primary. In recurrent infection, antibodies pass transplacentally to the fetus and protect against the infection. Passive transfer of VZV antibodies from mother to fetus protects the newborn from severe chickenpox when exposed days to weeks after delivery. Similarly, treating newborns with varicella zoster immune globulin greatly reduces the severity of infection in infants born too soon after onset of chickenpox in their mothers to benefit from passive transfer of maternal antibodies. Therefore, lacking mature T-cells, newborns rely on passive transfer of maternal IgG antibodies to modify disease severity, suggesting that antibodies are partially effective against herpesviruses. The immune evasion strategies of herpesviruses target the IgG Fc domain, but do not inhibit neutralizing activities mediated by the IgG Fab domain, which likely accounts for the partial protection provided by antibodies.

Role of the herpesvirus IgG Fc receptor in immune evasion

Introduction

Herpesviruses encode glycoproteins that bind the Fc domain of IgG, referred to as viral IgG Fc receptors (vFcγR). Table 63.1 lists the human herpesviruses that encode vFcγRs and the genes involved. Non-human herpesviruses, pseudorabies virus (PRV) and murine cytomegalovirus (MCMV), also express vFcγRs, suggesting that vFcγRs fulfill important roles in pathogenesis (Favoreel *et al.*, 1997; Thale *et al.*, 1994). FcγRs are detected on many microorganisms, including staphylococci (protein A), streptococci (protein G), schistosomes, trypanosomes, hepatitis C virus (core protein), and coronaviruses (S peplomer protein). FcγRs are also detected on mammalian cells (cFcγRs) and regulate B-cell activation, phagocytosis (engulfing particles $\geq$1 μM), endocytosis, antibody-dependent cellular cytotoxicity (ADCC), and release of inflammatory mediators (Raghavan and Bjorkman, 1996). Below, we discuss the structure and function of vFcγRs and consider similarities with cFcγRs.

The IgG Fc domain mediates important antibody effector activities, including C1q binding, interacting with cFcγR on NK cells to trigger ADCC, phagocytosis and release of cytokines and proinflammatory molecules from

Table 63.1. IgG Fc receptors encoded by human herpes viruses

Virus	Gene	Protein	Comments
HSV-1, HSV-2	U_S8	gE	Low affinity FcγR; homology with domain 2 of human FcγRII (Dubin *et al.*, 1994)
HSV-1, HSV-2	U_S8, Us7	gE-gI complex	gE-gI forms a higher affinity FcγR than gE alone (Dubin *et al.*, 1990).
CMV	U_L118, U_L119	gp68	Homology with domain 3 of FcγR1 (Atalay *et al.*, 2002)
CMV	TRL11, IRL11	gp34	Homology with FcγRII/III domain 2 (Atalay *et al.*, 2002)
VZV	ORF68	gE	VZV gE amino acids 328–500 shares homology with HSV-1 gE 211–381 (region involved in Fc binding) (Litwin *et al.*, 1992)

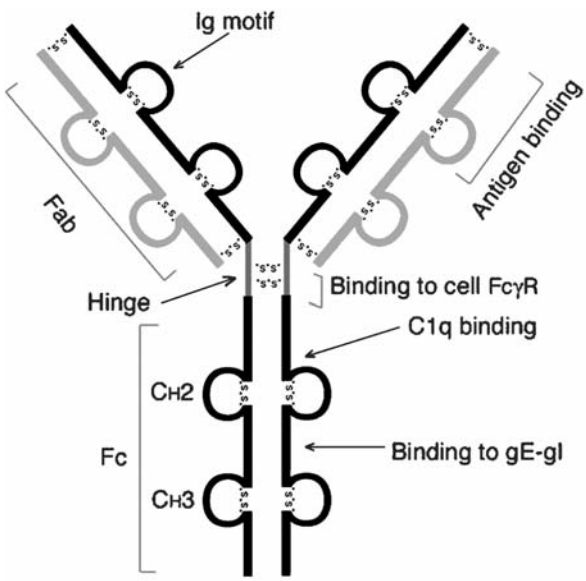

Fig. 63.1. Schematic drawing of the IgG molecule. Four immunoglobulin motifs are shown on the IgG heavy chains (black) and two on the IgG light chains (grey). Sites of interaction are shown between the IgG Fc domain and FcγRs on mammalian cells, C1q and HSV-1 gE-gI.

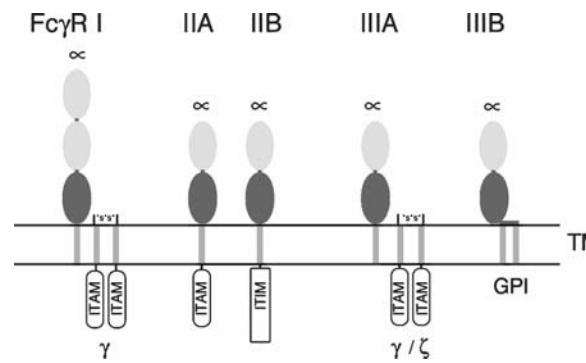

Fig. 63.2. Schematic drawing of cellular FcγRs. The FcγRs contain an α chain that has two or three Ig superfamily motifs (shown as ovals). The motif closest to the cell membrane (dark grey) functions as the IgG Fc binding domain. FcγR1 has two γ chains that contain ITAM (immunoreceptor tyrosine-based activation motif) sequences. FcγRII has two isoforms, IIA and IIB. IIA has an ITAM, while IIB has an ITIM (immunoreceptor tyrosine-based inhibitory motif) that inhibits activation signals. FcγRIII has two isoforms, IIIA that has ITAMs and IIIB that has glycophosphatidyl inositol (GPI) linkage.

granulocytes and macrophages. Figure 63.1 depicts an IgG molecule with its Fc domain showing the regions involved in C1q binding and interaction with cFcγRs or the HSV-1 vFcγR (gE-gI).

IgG Fc receptors on mammalian cells

Specific cellular Fc receptors interact with each of the immunoglobulin classes, IgA, IgD, IgE, IgG and IgM. However, only IgG Fc receptors have been detected on human herpesviruses; therefore, the discussion below focuses on cFcγRs.

Three classes of cFcγRs are present on mammalian cells, including FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (Fig. 63.2) (Raghavan and Bjorkman, 1996). FcγRI is detected on granulocytes (neutrophils, eosinophils and mast cells), monocytes and macrophages, and is a heterodimer consisting of one α-chain and two γ-chains. The α-chain contains three extracellular Ig-like domains. The membrane proximal domain is the region primarily involved in Fc binding activity. The γ-chains are linked by a disulfide bond and are necessary for cell signaling events and for expression of the α chain at the cell surface. The γ-chains contain amino acid sequences, referred to as ITAMs (immunoreceptor tyrosine-based activation motifs) that become phosphorylated at tyrosine positions upon cross-linking of the FcγR. The ITAMs trigger intracellular signaling events that are initiated by src family protein tyrosine kinases and that induce phagocytosis and endocytosis. FcγRI binds single IgG molecules (monomeric IgG)

with high affinity ($2 \times 10^9 - 5 \times 10^9$ M^{-1}). IgG complexes are required for efficient triggering of FcγRII and FcγRIII since these receptors bind monomeric IgG with low affinity ($\sim 10^6$ M^{-1}).

FcγRII has two human isoforms, IIA and IIB. Both contain a single α-chain with two Ig-like extracellular domains. The membrane proximal domain is involved in Fc binding activity. The cytoplasmic domain of FcγRIIA contains an ITAM motif, while IIB has an ITIM motif (immunoreceptor tyrosine-based inhibitory motif) that inhibits cell activation. Receptors with ITIMs are found on neutrophils, macrophages, mast cells and B cells and contain sequences that are phosphorylated at a tyrosine position upon receptor cross-linking leading to inhibition of activation signals (Ravetch and Bolland, 2001).

FcγRIII in involved in ADCC (NK cell-mediated), phagocytosis and endocytosis. This receptor has two isoforms, IIIA with a polypeptide chain anchor, and IIIB with a glycophosphatidyly inositol (GPI) linkage. FcγRIIIA contains an α chain with two Ig-like extracellular domains, and a γ- or ζ-chain that contains ITAMs. FcγRIIIB has only an α-chain with two extracellular Ig-like motifs. IgG Fc interacts with the membrane proximal Ig-like domains of both FcγRIII isotypes. FcγRIIIA is found on macrophages, mast cells and as the only FcγR on NK cells, where it mediates ADCC, while IIIB is detected on neutrophils.

Herpes simplex virus FcγR

Both HSV-1 and HSV-2 encode vFcγRs, although considerably more is known about the structure and functions of the HSV-1 FcγR, which is discussed below.

gE and gI structure

Glycoproteins gE and gI form a heterodimeric complex that contains one molecule each of gE and gI and that functions as an FcγR (Chapman *et al.*, 1999; Johnson *et al.*, 1988). HSV-1 gE strain 17 has a molecular mass of ~80 kDa and is a 550 amino acid type 1 transmembrane glycoprotein, although strain NS, used extensively in the authors' laboratory, encodes two additional amino acids, glycine and glutamine, at positions 186 and 187 respectively (Lin *et al.*, 2004). The NS strain gE ectodomain includes 421 amino acids with a predicted signal sequence from amino acids 1–23, two N-linked glycosylation sites, a transmembrane domain (422–446), and a large cytoplasmic tail (447–552) that undergoes serine phosphorylation (Edson *et al.*, 1987).

HSV-1 strain 17 gI has a molecular mass of ~70 kD and contains 390 amino acids. Sequences of strains KOS and NS differ from strain 17 in that a 7-amino acid repeat at position 225–231 in strain 17 is absent in KOS and NS (H. M. Friedman, unpublished observations). HSV-1 strain NS gI has a predicted signal sequence from amino acid 1–20, three N-linked glycosylation sites, a transmembrane domain (267–287), and a large cytoplasmic tail (288–383).

Glycoprotein gE binds Fc in the absence of gI, while gI cannot bind Fc without gE. The gE-gI complex binds Fc with higher affinity than gE alone (Bell *et al.*, 1990; Dubin *et al.*, 1990). IgG monomers bind to the gE–gI complex with an affinity of $0.4 \times 10^7 - 2 \times 10^7$ M^{-1}, while gE binds IgG aggregates, but not IgG monomers (Chapman *et al.*, 1999; Dubin *et al.*, 1990). Approximately 4×10^6 vFcγR binding sites are present on HSV-1 infected cells in vitro, which exceeds the number of cFcγR detected on human leukocytes by ~100-fold (Johansson and Blomberg, 1990). The HSV-1 FcγR binds human IgG4 with higher affinity than IgG1 or IgG2, while IgG3 fails to bind (Johansson *et al.*, 1984). A substitution of arginine for histidine at IgG4 Fc amino acid 435 abolishes Fc binding to gE-gI, which is consistent with the observation that most human IgG3 allotypes contain an arginine at Fc position 435 (Chapman *et al.*, 1999). IgG subclass concentrations in serum are age dependent, but in general, IgG1 is most abundant, followed by IgG2, with considerably lower concentrations of IgG3 and IgG4. IgG1 and IgG3 are potent activators of complement, while IgG2 is slightly less so, and IgG1 binds to FcγRIIIA on NK cells to mediate ADCC (Ghirlando *et al.*, 1995). Therefore, by interacting with IgG1 and IgG2, the vFcγR is binding the two most abundant IgG subclasses and potentially interfering with complement activation and ADCC mediated by these subclasses.

Mapping studies have determined that gE amino acids 24–211 are required to form a complex with gI, while gI amino acids 43–192 interact with gE (Basu *et al.*, 1995; Rizvi and Raghavan, 2001). Two approaches were used to define regions on gE involved in Fc binding. Fragments of gE DNA were fused to HSV-1 gD DNA and expressed in mammalian cells. The smallest gE fragment to retain FcγR activity included gE amino acids 183–402 (sequences based on strain 17) (Dubin *et al.*, 1994). Linker insertion mutagenesis was used as a second approach to evaluate gE domains involved in FcγR activity. Four amino acid inserts at each of ten positions between gE amino acids 235–380 eliminated IgG Fc binding. Therefore, the results of the two approaches were complementary, establishing the gE domain between amino acids 183 and 402 as sufficient for Fc binding, while mutations between amino acids 235 and 380 resulted in loss of function. The crystal structure has been solved for

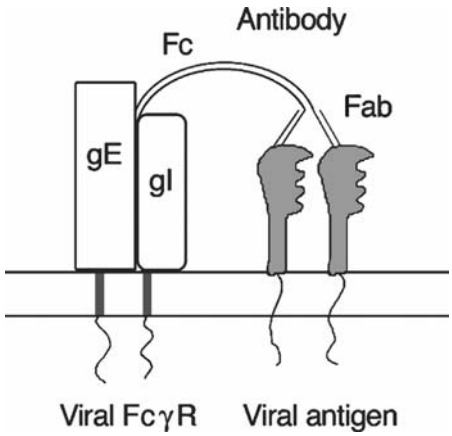

Fig. 63.3. Schematic drawing of antibody bipolar bridging. The IgG Fab domains bind to the viral antigen and the Fc domain of the same antibody molecule binds to the vFcγR (gE-gI).

the interaction of the IgG Fc fragment with a soluble form of cFcγRIII (Sondermann *et al.*, 2000). Five contact sites were identified on the cFcγR over a linear range of 73 amino acids, suggesting that the much broader linear range of gE mutations that resulted in loss of function likely reflect changes in conformation and contact sites. This conclusion is supported by a low-resolution crystal structure of the gE–gI/Fc complex that was solved at 5 Å (Sprague *et al.*, 2006). The crystal structure was verified by a theoretical prediction model of gE–Fc interaction that was based on the crystal structure of the gE C-terminal ectodomain (CgE amino acids 213–390) solved at 1.78 Å. The gE–gI/Fc crystal structure demonstrates that two gE-gI molecules interact with one Fc dimer and predicts that Fc interfaces with gE amino acids 225, 245–247, 249–250, 256, 258, 311, 316, 318–322, 324 and 338–342. Loss of Fc binding when gE is mutated at other sites likely occurs because of changes in gE conformation.

The HSV-1 FcγR and immune evasion

An IgG antibody molecule that is directed against HSV-1 can bind by its Fab domain to viral antigens on the virion or infected cell while the Fc domain of the same antibody molecule binds to the vFcγR (Fig. 63.3) (Frank and Friedman, 1989). Antibody bipolar bridging is used to describe this form of antibody binding, which requires considerable flexibility of the IgG molecule at the hinge region. Studies of the dynamic conformations of IgG in solution and bound to receptors confirm IgG has the flexibility to mediate bridging (Zheng *et al.*, 1992). The crystal structure of the gE–gI/Fc complex is also compatible with antibody bipolar bridging (Sprague *et al.*, 2006). Antibody bridging is postulated to be an important immune evasion strategy, since the vFcγR binds the Fc domain of antibody molecules that are targeting the virus. The affinity of the HSV-1 FcγR for Fc is ~100-fold lower than that of cFcγR1; therefore, binding of monomeric, non-immune IgG is limited. The IgG Fab domain binds with high affinity to the target antigen, which anchors the IgG Fc domain onto the virion or infected cell surface. The vFcγR is then able to bind the IgG Fc domain to block its activities.

Figure 63.1 shows the regions on Fc that interact with host and viral proteins. The region on IgG that binds to cFcγRs is located at the lower portion of the hinge region and the upper margin of the C_H2 domain (Sondermann *et al.*, 2000). The IgG Fc C_H2 domain interacts with C1q to initiate complement activation, while the C_H2–C_H3 interface of the Fc domain binds to gE–gI, which is similar to the site of interaction between protein A and Fc (Johansson *et al.*, 1989; Miletic and Frank, 1995). Despite the distance between domains on Fc involved in binding to gE–gI, C1q and cFcγRs, the HSV-1 FcγR is effective at blocking functions mediated by these regions of the Fc molecule (Dubin *et al.*, 1991).

Studies were performed to assess the role of the vFcγR in pathogenesis. A mutant HSV-1 strain was produced by introducing 4 amino acids at gE position 339 (based on the sequence of strain 17). The mutant virus expressed gE and gI at the infected cell surface, but failed to bind IgG Fc measured by rosetting assays using IgG-coated erythrocytes and by flow cytometry using biotin-labeled non-immune human IgG (Nagashunmugam *et al.*, 1998). Wild-type, gE mutant and gE-restored viruses were injected into the murine flank to assess the contribution of the vFcγR to virulence. The Fc domain of murine IgG does not bind to the HSV-1 FcγR; therefore, the three virus strains were expected to cause similar disease in mice, which was the observed result (Johansson *et al.*, 1985; Nagashunmugam *et al.*, 1998). Human IgG does bind to the HSV-1 FcγR; therefore, passive transfer of HSV antibodies was predicted to be more active against the vFcγR defective strain than against wild-type or restored virus. When infection was performed one day following passive transfer of HSV IgG antibodies, approximately 100-fold higher titers of gE mutant virus were required to cause the same level of disease as wild-type or restored virus. Passive transfer of non-immune human IgG or murine HSV IgG antibodies showed no differences among the various strains. Therefore, these in vivo studies define a contribution of the vFcγR to pathogenesis that depends upon the ability of the virus to block activities mediated by the IgG Fc domain. Synergy between gC and gE in mediating immune evasion was demonstrated

in vitro and in vivo using an HSV-1 mutant virus defective in C3b and IgG Fc binding (Lubinski *et al.*, 2002). Glycoproteins gC and gE interfere with complement activation at different steps in the cascade, which likely contributes to the synergy.

Additional mechanisms have been proposed for vFcγR-mediated immune evasion. The vFcγR promotes capping of viral antigens on the surface of infected cells in response to human HSV antibodies (Rizvi and Raghavan, 2003). The antibodies promote virus spread cell-to-cell when gE is expressed on the cell surface, which suggests that when antibodies are present, gE enables the virus to remain intracellular to avoid neutralization. In separate studies, the binding of Fc to gE-gI was noted to be pH dependent (Sprague *et al.*, 2004). Binding of Fc occurs at an affinity of $2 \times 10^7 - 3 \times 10^7$ M^{-1} at pH 7.4, which is the pH at the cell surface. In contrast, no binding occurred at pH 6.0. Based on antibody internalization studies described below, the results suggest that the vFcγR may internalize IgG and then dissociate within acidic intracellular compartments promoting degradation of antibodies. The crystal structure of the gE–gI/Fc interaction predicts that histidines at gE position 247 and Fc positions 310 and 435 are likely involved in Fc dissociation from gE at acidic pH (Sprague *et al.*, 2006).

Human CMV FcγR

Cells infected with human CMV (HCMV) express $\sim 10^6$IgG Fc receptors per cell that have an association constant of 2×10^8 M^{-1} (Antonsson and Johansson, 2001). Two HCMV vFcγRs have been reported (Atalay *et al.*, 2002; Lilley *et al.*, 2001). The first is a 234-amino acid type 1 transmembrane glycoprotein that migrates with an apparent molecular mass of 34 kDa (gp34) and has three potential N-linked glycosylation sites in the ectodomain (Lilley *et al.*, 2001). The glycoprotein is encoded by TRL11 and IRL11, which are identical copies of a gene found in the terminal and internal repeats of the HCMV DNA long fragment. The cytoplasmic tail of gp34 has 31 amino acids and contains a conserved dileucine motif that is postulated to participate in IgG endocytosis. Sequence similarities were detected between gp34 and domain 2 of cFcγRII and III (Atalay *et al.*, 2002). The other HCMV FcγR is encoded by the UL119–118 open reading frame that produces a 347 amino acid type 1 transmembrane glycoprotein with a molecular mass of 68 kDa (gp68) (Atalay *et al.*, 2002). The glycoprotein has 12 potential N-linked sites and an immunoglobulin supergene family-like variable domain that shares sequence homology with cFcγRI domain 3 (Atalay *et al.*, 2002). The cytoplasmic tail has a possible modified ITIM-like motif (WSYKRL) that may be involved in cell signaling events. The lack of laboratory animal models for HCMV has hampered attempts to define the role of the HCMV FcγRs in pathogenesis.

Varicella zoster FcγR

The VZV US8 gene encodes gE, which functions as an FcγR on infected human cells (Litwin *et al.*, 1992). Glycoprotein gE is a 623-amino acid type 1 transmembrane glycoprotein that has a signal sequence of 24 amino acids, a 544-amino acid extracellular domain with three predicted N-linked glycosylation sites, a 17-amino acid transmembrane domain and a 62 amino acid cytoplasmic tail. Binding of IgG Fc to gE initiates endocytosis, unloading the IgG cargo in lysosomal vesicles and subsequent recycling of gE to the cell surface (Olson and Grose, 1997). Endocytosis requires the gE cytoplasmic tail, and is mediated by tyrosine phosphorylation of a YAGL endocytosis motif (Olson and Grose, 1997). VZV gE shares sequence similarities with HSV gE, but not with cFcγRs (Litwin *et al.*, 1992). Similar to HCMV, the in vivo relevance of the VZV FcγR in immune evasion has not been determined because of the lack of animal models.

vFcγRs on non-human mammalian herpesviruses

Murine CMV

The m138 (fcr-1) gene of MCMV encodes a vFcγR that has a molecular mass of 88 kDa and is a 569 amino acid type 1 transmembrane glycoprotein. The protein has a predicted signal sequence of 17 amino acids, a transmembrane domain from amino acids 535–552, 10 potential N-linked glycosylation sites, and a 17 amino acid cytoplasmic tail (Thale *et al.*, 1994). Studies were performed to assess the function of the vFcγR in its natural host by preparing a mutant virus deficient in the fcr-1 gene and a revertant strain (Crnkovic-Mertens *et al.*, 1998). The fcr-1 deficient strain showed normal replication kinetics in vitro, but significantly reduced replication in vivo. To determine if the reduced replication in vivo was caused by increased susceptibility to antibody, mutant and revertant strains were injected into B cell deficient mice. The expectation was that the two strains would have similar replication patterns in antibody deficient mice; however, this did not occur, suggesting that the vFcR had little or no role in pathogenesis. While this conclusion is potentially correct, other explanations are also possible. For example, to demonstrate a

role for the HSV-1 FcγR in pathogenesis, small deletions were made in gE to abolish Fc binding without interfering with virus spread, another activity mediated by gE (Nagashunmugam *et al.*, 1998). A similar approach may be required to assess the potential role of the MCMV vFcγR in pathogenesis.

Pseudorabies virus

PRV gE and gI form a molecular complex that functions as a vFcγR (Favoreel *et al.*, 1997). PRV US8 encodes gE, a 62 kD type 1 transmembrane glycoprotein containing 577 amino acids, including an extracellular domain of 428 amino acids, a transmembrane domain of 26 amino acids, and a cytoplasmic tail of 123 amino acids (Klupp *et al.*, 2004). PRV US7 encodes gI, which is a type 1 transmembrane glycoprotein containing 366 amino acids, including an extracellular domain of 285 amino acids, a transmembrane domain from amino acid 286–308, and a cytoplasmic tail of 58 amino acids (Klupp *et al.*, 2004).

Studies performed in swine kidney cells demonstrate that PRV-specific antibodies induce capping of viral glycoproteins followed by their extrusion from the cell surface (Favoreel *et al.*, 1997). Phosphorylation of two tyrosine motifs in the gE cytoplasmic tail are required for glycoprotein capping (Favoreel *et al.*, 1999). Studies of PRV in swine monocytes, which are infected by PRV during natural infection, demonstrate that antibodies induce endocytosis of viral glycoproteins with co-internalization of MHC class I proteins (Favoreel *et al.*, 2003). Endocytosis removes viral glycoproteins from the cell surface and reduces the effectiveness of antibody dependent complement lysis of infected cells. Endocytosis is not uniquely mediated by gE-gI, but the vFcγR contributes to this activity (Van de Walle *et al.*, 2003).

Summary of vFcγR studies

Activities mediated by the vFcγRs of human and non-human herpesviruses can be summarized as follows. First, antibody bipolar bridging is an important mechanism used by vFcγRs to protect the virus and infected cell against activities mediated by the IgG Fc domain, including complement activation and ADCC. Second, some vFcγRs mediate antibody capping, while others promote antibody internalization. In acidic intracellular compartments, antibodies may dissociate from the vFcγR and degrade, while the vFcγR recycles to the cell surface. Third, potential ITIM motifs have been noted on some vFcγRs that may regulate responses to antibody. Fourth, the HSV-1 FcγR is a virulence factor, reducing the effectiveness of antibodies in vivo.

Role of the herpesvirus complement receptors in immune evasion

Introduction

The complement system plays an important role in both the innate and adaptive immune responses to viral infection. Activation of complement early following viral infection relies on the presence of highly specific recognition proteins, which have evolved to recognize and bind pathogen associated molecular patterns (PAMPs). These pattern recognition proteins include natural antibodies (IgM), C1q, C-reactive protein, mannan-binding lectin, and ficolins H and L. IgG and IgM antibodies are able to trigger the activation of complement following induction of specific humoral immune responses.

Complement is activated by one of three different pathways: classical, mannan-binding lectin (MBL), or alternative (Fig. 63.4). The classical complement pathway was the first pathway to be identified, and is normally considered to be antibody-dependent. Activation of the classical pathway occurs when the first component of the pathway, C1, binds the Fc region of either natural antibody or specific IgG antibody in complex with viral antigen. The classical pathway is also triggered in an antibody-independent manner when C1 binds directly to virions or infected cells.

Activation of the MBL pathway is antibody-independent and occurs when one of the C-type lectins, MBL, ficolin H, or ficolin L recognizes carbohydrate structures on the surface of pathogens. The alternative complement pathway was originally described as the antibody-independent pathway (the MBL pathway was identified much later). Activation of the alternative pathway is spontaneous as a continued low-level release of the internal thioester bond of C3 allows it to bind to a wide range of "foreign" sites. The diversity of serum recognition proteins able to recognize and activate complement allows the complement system to protect against a wide variety of microbial pathogens.

Recently a fourth pathway for complement activation was described that involves SIGN-R1, a C-type lectin detected on marginal-zone macrophages in the spleen. The unique feature of this pathway is that SIGN-R1 binds C1q and activates the classical complement pathway in the absence of immunoglobulins. SIGN-R1 also binds the capsular polysaccharide of *S. pneumoniae*, and perhaps carbohydrates on other microbial pathogens, leading to C3 deposition on the organism and enhanced innate resistance to infection (Kang *et al.*, 2006).

Activation of the complement cascade leads to numerous effector functions that result in neutralization and elimination of virus, thereby limiting spread, infection and disease. These include neturalization of viral particles, phagocytosis of complement-opsonized virus and virus-infected cells, direct lysis of virus and infected cells by the formation of pores known as the membrane attack complex, induction of inflammation, and enhancement of the adaptive immune response.

Regulation of complement

Given the complexity of the complement system, with over 30 complement proteins involved in activating the three divergent complement pathways, proper control is imperative. Regulation of the complement systems is necessary to prevent inappropriate activation and injury to bystander cells. Therefore, complement is tightly regulated by proteins present in serum and expressed on the surface of cells. These complement regulatory proteins include C1 inhibitor, CD59, and a class of proteins referred to as regulators of complement activation (RCA). RCA proteins include secreted plasma factor H, C4-binding protein and membrane bound complement regulatory proteins 1, 2, 3 (CR1, CR2, CR3), membrane cofactor protein (MCP) and decay accelerating factor (DAF) (Carroll, 2000; Da Costa *et al.*, 1999; Spear *et al.*, 1995; Spiller *et al.*, 1997). Both CR2 and DAF are GPI-linked.

RCA proteins are homologous in structure, characterized by the presence of motifs known as short consensus repeats (SCRs). SCR motifs contain approximately 58 to 66 amino acids, with four conserved cysteine residues disulfide linked (cys 1 to 3, and cys 2 to 4) and several hydrophobic residues. SCR motifs are highly conserved, sharing 30%–40% amino acid identity. However, the number of SCRs contained within RCA proteins is highly variable, for example, both MCP and DAF contain four SCRs, while CR1 contains 30.

As the complement system plays an important role in host defense against viral infection, not surprisingly, viruses have evolved numerous mechanisms to control complement. Strategies employed by viruses fall into three categories: (1) viral proteins which are homologous to mammalian complement regulatory proteins; (2) viral proteins which have no sequence homology, but which share functional characteristics with complement regulatory proteins; and (3) viruses that incorporate host complement regulatory proteins into their envelope during viral maturation and egress.

Strategies employed by human herpesviruses to evade complement immunity

Viral proteins homologous to human complement regulatory proteins: Kaposi's sarcoma associated herpesvirus complement control protein

Sequencing of the Kaposi's sarcoma associated herpesvirus (KSHV) genome indicated that one gene, KSHV open reading frame 4 (ORF4), encoded a protein displaying a high degree of homology to many complement regulatory proteins, including RCA proteins DAF and MCP (Neipel *et al.*, 1997; Russo *et al.*, 1996). The KSHV ORF4 was predicted to encode a protein of 550 amino acids residues, with the first 270 amino acids forming four SCRs sharing 24.7% identity with DAF (Spiller *et al.*, 2003b).

Characterization of transcripts produced by KSHV ORF4 indicated that three transcripts were produced, one full length, and two smaller, alternatively spliced forms (Spiller *et al.*, 2003b). All three protein isoforms contained the four amino-terminal SCRs and transmembrane regions, not a GPI anchor present in DAF, and collectively, are referred to as KSHV complement control protein (KCP) or kaposica (Mullick *et al.*, 2003; Spiller *et al.*, 2003b).

Functional studies indicated that all three forms prevented C3 deposition on cell surfaces (Spiller *et al.*, 2003b). Addition of a soluble form of each KCP isoform accelerated the decay of the classical pathway C3-convertase (Spiller *et al.*, 2003a). All three forms of soluble KCP also accelerated the decay of the alternative pathway C3-convertase; however, they were 1000-fold less efficient than DAF, indicating that KCP mainly affects the classical pathway C3-convertase.

KCP acts as a cofactor for factor I (fI) mediated cleavage and inactivation of C3b and C4b (Mullick *et al.*, 2003; Spiller *et al.*, 2003a). KCP results in fI mediated cleavage of C4b to C4d, when both soluble and cell bound. KCP also acts as a cofactor for fI mediated cleavage of C3b to iC3b, and induces cleavage of iC3b to C3d, the only viral protein shown to date to do so. Binding affinity of KCP is higher for C4b than C3b, which may explain differences in the ability of KCP to degrade the classical and alternative pathway C3-convertases.

Viral proteins with no sequence homology, yet functional similarities with human complement regulatory proteins

HSV-1 and HSV-2 glycoprotein gC

HSV-1 and HSV-2 encode gC, a viral complement control protein that inhibits complement activation by

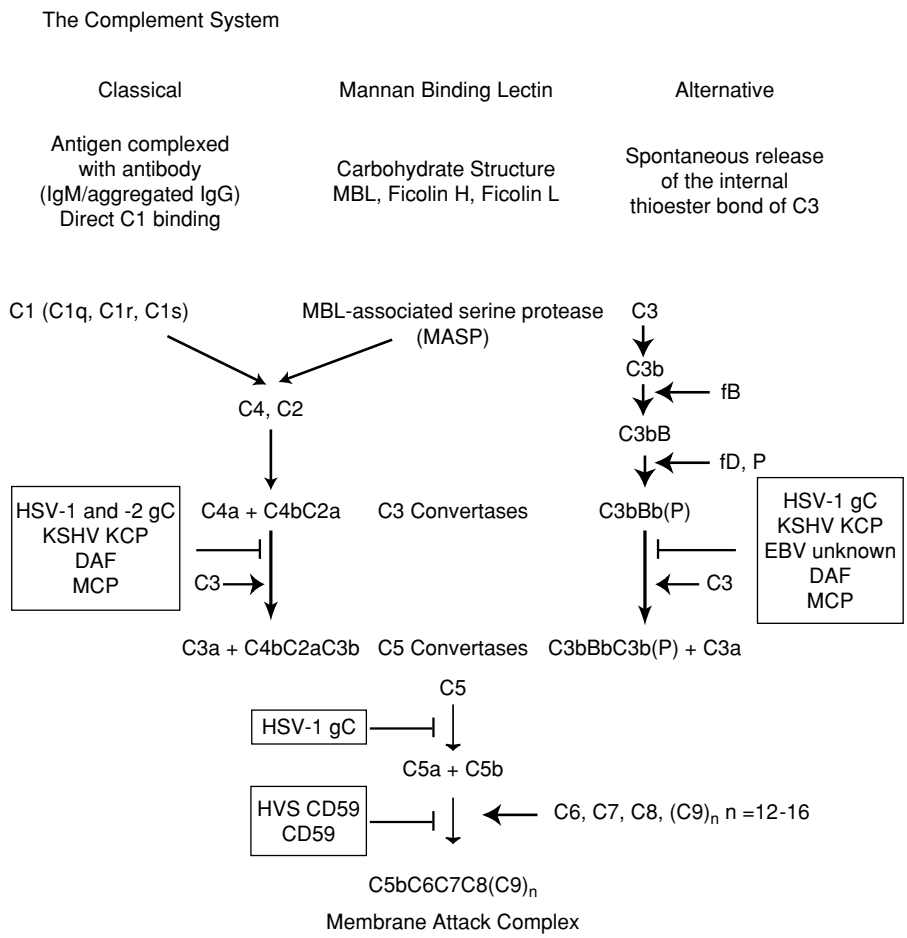

Fig. 63.4. Sites of interaction between herpesviruses and the complement cascade. The boxed type identifies the sites in the complement cascade inhibited by viral proteins or by host-derived cellular proteins captured by viruses.

binding C3b (Friedman *et al.*, 1984; Kostavasili *et al.*, 1997). gC of HSV-1 (gC-1) was the first complement control protein identified, and has been extensively characterized. Both gC-1 and gC-2 are rarely absent from clinical isolates, underscoring their importance *in vivo*. Moreover, gC is well conserved among members of the alpha-herpesvirus family, with homologues present in VZV, PRV, bovine herpesvirus 1 (BHV-1), and equine herpesviruses 1 and 4 (EHV-1, EHV-4). Despite this high degree of sequence conservation among alphaherpesviruses, gC displays little sequence homology with known complement regulatory proteins.

gC-1 and gC-2 are encoded by the UL44 gene and are type I membrane glycoproteins of 511 and 480 amino acids respectively, which are expressed on virions and infected cells. Both are highly glycosylated with 9 and 7 N-linked glycosylation sites respectively (Rux *et al.*, 1996). gC-1 also contains several O-linked glycosylation sites, localized to the amino terminus of the protein; gC-2, however, is not O-linked glycosylated. gC-1 and gC-2 share eight highly conserved cysteines, the disulfide bonding pattern of which has been determined for gC-1, and is likely similar for gC-2 and the gC homologues from other herpesviruses (Rux *et al.*, 1996).

gC of both HSV-1 and HSV-2 binds C3b in a purified form and when expressed on the surface of transfected cells (Eisenberg *et al.*, 1987; Tal-Singer *et al.*, 1991). However, only HSV-1 infected cells express gC that is able to bind C3b (Friedman *et al.*, 1984). Cells infected with HSV-2 display no C3b receptor activity (Friedman, 1986; Friedman *et al.*, 1984). Lack of binding to C3b appears unrelated to affinity of gC-2 for C3b, as optical biosensor technology indicates that gC-2 has a tenfold higher affinity for C3b compared with gC-1 (Rux *et al.*, 2002). One possible explanation may be that other host cell membrane components or viral glycoproteins expressed on the HSV-2 infected cell surface may interfere with the ability of gC-2 to bind C3b.

Both gC-1 and gC-2 bind C3 and its activation products C3b, iC3b and C3c (Kostavasili *et al.*, 1997; Tal-Singer *et al.*, 1991). This binding is mediated by C3b regions, which are well conserved in both glycoproteins. Four regions in gC-1 and three in gC-2 were identified by site-directed and linker insertion mutagenesis, with binding phenotypes confirmed in rosetting assays using C3b-coated sheep erythrocytes (Hung *et al.*, 1992; Seidel-Dugan *et al.*, 1990).

Functional studies indicate that gC-1 prevents complement-mediated neutralization of HSV-1 by binding C3b, thereby inhibiting activation of the classical complement pathway (Friedman *et al.*, 1996; Harris *et al.*, 1990). Evidence suggests that gC-2 may function in a similar manner (Gerber *et al.*, 1995). Neutralization of HSV-1 in the absence of gC-1 is mediated by a C5 dependent mechanism that does not require viral lysis, aggregation, or prevention of viral attachment (Friedman *et al.*, 2000). Complement likely interferes with HSV infection at a stage following viral attachment, for example, during virus entry or uncoating (Friedman *et al.*, 2000).

gC-1 also prevents complement-mediated cell lysis of HSV-1 infected cells by accelerating the decay of the alternative pathway C3 convertase, C3bBb (Fries *et al.*, 1986; Harris *et al.*, 1990). The half-life of the C3bBb complex is extended three- to four-fold by the binding of properdin to C3b in the convertase. gC-1 contains a properdin interacting domain, localized to the amino-terminus, which interferes with the binding of properdin to C3b, destabilizing the C3 convertase (Hung *et al.*, 1994; Kostavasili *et al.*, 1997). By interfering with properdin's ability to bind C3b, gC-1 prevents activation of the alternative complement pathway. This prevents lysis of HSV-1 infected cells.

gC-1 also contains a C5 interacting domain which prevents C5 from binding C3b (Fries *et al.*, 1986). gC-1 thus interferes with activation of both the classical and alternative complement pathways, limiting neutralization of both virus and lysis of virus-infected cells. While both gC of HSV-1 and HSV-2 prevent complement-mediated neutralization of HSV virions, differences do exist. Only gC-1 is able to disrupt the activation of alternative pathway C3 convertase (Fries *et al.*, 1986; Kostavasili *et al.*, 1997). Moreover, the region of gC-1 important in blocking the binding of C5 and properdin to C3b is absent in gC-2.

Studies comparing the importance of both complement interacting domains of gC-1 were evaluated both in vitro and in an in vivo model of HSV pathogenesis (Lubinski *et al.*, 1999). Complement neutralization experiments performed with a low passage clinical isolate that had been mutated within the C3 binding domain, the C5 and properdin blocking domain, or both, indicated that while both domains were important, elimination of the C3 binding domains significantly diminished the ability of gC to modulate complement (Lubinski *et al.*, 1999). Similar results were seen in vivo in a murine model of HSV pathogenesis. HSV-1 was mutated in the C5 and properdin blocking, the C3 binding, or both domains (double mutant). Each mutant virus was significantly more attenuated than the wild type HSV-1 virus (Lubinski *et al.*, 1999). That the C3 binding domain mutant was as attenuated as the gC double mutant indicated that the C3 domain is more important than the C5 and properdin in modulating complement activity.

EBV complement regulatory activity: unidentified protein

In addition to gC of HSV-1 and HSV-2, evidence suggests that Epstein–Barr virus (EBV) encodes a complement regulatory protein displaying no sequence homology with known human complement regulatory proteins. EBV virions derived from either marmoset or human B lymphoblastoid cells maintains complement regulatory activities (Mold *et al.*, 1988). Incubation of purified EBV with immune human serum resulted in the cleavage of C3 into the inactive C3c. In addition, EBV functions as a cofactor for the Factor I mediated cleavage of C3b and iC3b and C4b and iC4b. No degradation occurred in the absence of Factor I. EBV accelerates the decay of the alternative, but not the classical C3 convertase. The EBV envelope protein responsible for this complement regulatory activity remains unknown. No virally encoded proteins with homology to known complement regulatory proteins have been identified; therefore, cellular proteins incorporated into the virion are possibly mediating this effect.

Viruses that incorporate human complement regulatory proteins into their envelope during viral maturation and egress: human CMV

HCMV-infected cells remain susceptible to antibody-mediated complement cytolysis for only a brief time following acute infection, suggesting that the cells are protected from complement lysis. Analyses of the complete genomic sequence of HCMV revealed no homologues of known complement regulatory proteins, and HCMV does not appear to encode a C3 binding protein (Smiley and Friedman, 1985). It was hypothesized that HCMV may alter the expression of host-encoded complement regulatory proteins in order to interfere with complement (Spiller *et al.*, 1996). Candidates included DAF, MCP, and CD59, since each are expressed on uninfected cells that are permissive to infection by HCMV.

Studies examining the cell surface expression of both DAF and MCP by HCMV infected human foreskin fibroblasts indicated that levels were enhanced at 24, 48,

and 72 hours post infection compared with mock-infected controls. Maximal expression was seen 72 hours post infection with a 3.4-fold and 8-fold increase in MCP and DAF respectfully. Expression of CD59, however, remained relatively stable (Spiller *et al.*, 1996).

Incubation of HCMV virions with complement alone consumed complement activity and resulted in C3 deposition on the surface of the virion, yet resulted in negligible amounts of C9 deposition and no loss of viral infectivity (Spiller *et al.*, 1997). These data suggest that HCMV is able to regulate complement and accomplishes this by interfering with the complement system upstream of C9 activation. Studies examining the expression of MCP, DAF, and CD59 on HCMV virions produced in human foreskin fibroblasts indicated that the three complement regulators were captured by the virion during egress and maturation (Spear *et al.*, 1995; Spiller *et al.*, 1997).

The mechanism by which MCP, DAF, and CD59 become incorporated within the HCMV virion remains unknown. Incorporation could represent a passive capture of upregulated plasma membrane proteins, as levels of both DAF and CD59 are increased during HCMV infection and are readily incorporated into foreign membranes (Spiller *et al.*, 1996). Moreover, the distribution of both DAF and CD59 expressed on the surface of the virion correlates roughly with levels of each detected on the surface of the host derived cells, indicating that virions may obtain the host cell derived complement regulatory proteins in a passive manner (Spear *et al.*, 1995). Incorporation, however, could be an immune evasion strategy adopted by HCMV, in order to protect virions and virus infected cells from complement-mediated neutralization. Treatment of HCMV virus with an anti-DAF antibody, not anti-CD59, reduced HCMV infectious titer in the presence of complement, indicating that DAF interferes with complement-mediated neutralization of HCMV virus (Spear *et al.*, 1995).

Strategies employed by non-human mammalian herpesviruses to evade complement immunity

Viral proteins homologous to mammalian complement regulatory proteins

Murine Gammaherpesvirus 68: MHV-68 RCA

Murine Gammaherpesvirus 68 (γHV68) gene 4 product is a complement regulatory protein, with significant homology to both virally encoded and cellular proteins, including the herpesvirus saimiri complement control protein homologue (CCPH), DAF, and MCP (Virgin *et al.*, 1997). γHV68 ORF 4 includes four regions with homology to SCRs of RCA complement regulatory proteins. γHV68 gene 4, named γHV68 RCA, produces a 5.2 kb bicistronic mRNA of the late kinetic class, encoding multiple γHV68 RCA proteins, including both plasma membrane bound and soluble forms (Kapadia *et al.*, 1999).

Functional studies indicate that γHV68 RCA interferes with both murine and human complement activation, resulting in a decrease in C3b deposition (Kapadia *et al.*, 1999). γHV68 RCA was found to prevent activation of both the classical and alternative complement pathways (Kapadia *et al.*, 1999).

The γHV68 RCA contributes to virulence in mice, as γHV68 virus mutated within the RCA protein was more attenuated during both acute and persistent γHV68 infection when compared with wild type or marker rescued virus (Kapadia *et al.*, 2002). γHV68 RCA accomplishes this by interfering with the complement system, as the virulent phenotype of the γHV68 RCA protein mutant virus was restored in mice lacking C3. Interestingly, γHV68 RCA was not involved in evading C3 mediated innate immunity during latent infection.

Herpesvirus saimiri (HVS): complement control protein homologue (CCPH)

While determining the nucleotide sequence of genes within the vicinity of STP-A and STP-C (saimiri transformation associated protein of subgroup A and C), a gene was detected that encodes a protein displaying a significant degree of homology to the RCA protein family (Albrecht and Fleckenstein, 1992; Albrecht *et al.*, 1992a). This protein, named complement control protein homologue (CCPH), is encoded by HVS 04, and contains four SCRs within its N-terminal domain (amino acids 21–265), seven potential amino-linked glycosylation sites, and a transmembrane domain.

HVS 04 was found to encode two transcripts, one full length, and one smaller, alternatively spliced form. The unspliced transcript encodes a membrane-bound glycoprotein (mCCPH) of 65–75 kD, similar to the membrane-bound RCAs MCP, DAF, CR1, and CR2. Splicing produced a secreted glycoprotein (sCCPH) of 47–53 kD, like the soluble complement inhibitors C4-binding protein and Factor H. Functional studies indicated a role for CCPH in complement regulation. Cells stably transfected with mCCPH were approximately two times more resistant to complement mediated cell lysis, with levels similar when compared with DAF as a control (Fodor *et al.*, 1995).

HVS: CD59

In addition to HVS CCPH, sequence analyses of the HVS genome indicated the presence of a second gene, HVS 15

that encodes a protein sharing significant homology with the complement regulatory protein CD59 (Albrecht *et al.*, 1992b). The HVS 15 ORF consists of 363 nucleotides, sharing 64% sequence identity with human CD59 cDNA and was predicted to encode a protein of 121 amino acids with 48% identity to human CD59. Both HVSCD59 and CD59 have hydrophobic carboxyl-terminal sequences, which for CD59, is replaced by a GPI anchor. Additional studies confirmed that HVS 15 protein product was also a GPI linked membrane glycoprotein (Rother *et al.*, 1994). The overall structure of both HVS 15 and CD59 were expected to be very similar as the proteins shared amino acid identities and a single N-linked glycosylation site, and all cysteines were highly conserved.

Expression of either HVSCD59 or squirrel monkey CD59 (SMCD59), the natural host of HVS, on the surface of Balb/3T3 cells rendered the cells resistant to complement-mediated cell lysis by human serum (Rother *et al.*, 1994). However, only HVSCD59 expressing cells were protected from challenge with rat serum, indicating that HVSCD59 is less species specific than either human or SMCD59. Protection occurred following C3b deposition, suggesting that HVSCD59 prevents complete formation and function of the membrane attack complex (Rother *et al.*, 1994). It was hypothesized that the location of the N-linked glycosylation site was responsible for the less species restrictive phenotype of HVSCD59, as the N-linked glycosylation of human CD59 appears necessary for its function.

Viral proteins with no sequence homology, yet functional similarities with mammalian complement regulatory proteins, and that incorporate host complement regulatory proteins: PRV gC and unknown host cell derived complement regulatory protein(s)

Pseudorabies virus (PRV) is protected from complement-mediated innate immunity by the presence of at least two proteins able to interfere with complement, gC and one or more host cell derived complement regulatory proteins. PRV gC shares homology with gC from other members of the alpha-herpesvirus family, and like HSV-1 gC, mediates viral attachment and facilitates immune evasion by binding C3. Neutralization experiments comparing PRV-CPK, a virus containing both gC and host cell derived complement regulatory proteins, and PRV-ΔgC-CPK which expresses the host cell derived complement regulatory proteins, yet lacks gC were performed (Maeda *et al.*, 2002). The PRV virus lacking gC was more readily neutralized by swine serum than the wild type virus, suggesting that gC protects PRV from complement-mediated neutralization.

Pseudorabies virus (PRV) in which gC was deleted was propagated on either swine kidney derived CPK or rabbit kidney derived RK13 cells and then tested for susceptibility to complement-mediated virus neutralization using either swine or rabbit serum as the source of complement (Maeda *et al.*, 2002). Results indicated that the gC deletion mutant grown in the CPK porcine cells was protected from neutralization mediated by swine serum. However, PRV derived from the RK13 rabbit cells was susceptible, suggesting that in the absence of gC, PRV was protected by at least one additional complement regulatory protein. No known homologues of complement regulatory proteins exist within the PRV genome; therefore, a host cell derived complement regulatory protein likely confers protection. Additional studies indicated that while the greatest level of protection was obtained when both gC and the cell derived factor(s) were coexpressed, the cell derived factor(s) afforded the most protection.

Summary of viral complement regulatory proteins

The complement system is an early innate defense able to thwart viral infection. Viruses have evolved numerous mechanisms to interfere with complement that are summarized in Fig. 63.4. Strategies reflect those used by the host to regulate and control complement activation and include encoding complement regulatory proteins that can be secreted or expressed on the surface of virions and infected cells or up-regulating and incorporating the host's own complement regulatory proteins on infected cells and within virions. Expression of complement control proteins by viruses can impart the capacity for increased infection and spread, resulting in greater virulence.

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Part VI

Antiviral therapy

Edited by Ann Arvin and Richard Whitley

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Antiviral therapy of HSV-1 and -2

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Introduction

The discovery of effective antiviral agents has been facilitated by advances in the fields of molecular biology and virology. In the pre-antiviral era, the widely held belief was that any therapeutically meaningful interference with viral replication would destroy the host cell upon which viral replication was dependent. A growing understanding of host cell–virus interactions and viral replication, however, has led to the development of safe and effective antivirals. These agents act by impeding entry of viruses into host cells; interfering with viral assembly, release, or deaggregation; inhibiting transcription or replication of the viral genome; or interrupting viral protein synthesis.

Antiviral agents can be used to treat disease (a therapeutic strategy), to prevent infection (a prophylactic strategy), or to prevent disease (a preemptive strategy). Prophylaxis refers to the administration of an agent to patients at risk of contracting infection (e.g., acyclovir given to HSV-seropositive renal transplant recipients). Pre-emptive treatment refers to the administration of a drug after there is evidence of infection, but before there is evidence of disease (e.g., ganciclovir given to bone marrow transplant recipients with positive CMV culture, but no symptoms of infection).

The effectiveness of antiviral therapy sometimes is limited by the development of antiviral resistance. Antiviral drug resistance has increased in parallel with the expanded use of, and indications for, antiviral therapy. Resistance most commonly occurs in patients with chronic and/or progressive infections who have been exposed to prolonged or repeated courses of therapy. An impaired host immune system which cannot fully contribute to suppressing viral replication, thus leaving to antiviral agent(s) as the sole defense against ongoing viral disease, also predisposes to the development of antiviral resistance, as does administration of the antiviral agent at doses which produce subtherapeutic drug concentrations relative to that needed for virocidal or virostatic activity. In general, antiviral resistance should be suspected if the clinical response to therapy is less than that anticipated on the basis of prior experience (Kimberlin *et al.*, 1995b).

There are a number of antiviral medications with activity against HSV-1 and HSV-2. With the exception of foscarnet and cidofovir, all are nucleoside analogues. While three of these medications (acyclovir, famciclovir, and valaciclovir) are used to treat the overwhelming majority of cases of HSV-1 and HSV-2, the other medications reviewed in this chapter (cidofovir, foscarnet, ganciclovir, and valganciclovir) also have activity against the alpha herpesviruses and are indicated in certain circumstances, such as the treatment of some acyclovir-resistant HSV isolates. Discussion in this chapter of the efficacies of these first-line and second-line drugs will be limited to their use as second-line agents for HSV-1 and HSV-2 infections. Antiviral treatment of VZV and CMV can be found in Chapters 70 and 71, respectively.

First-line antiviral agents for HSV-1 and HSV-2 infections

Acyclovir

Acyclovir is in many regards the prototypic antiviral agent. The notable safety profile of acyclovir relates to its initial activation by the viral-induced enzyme thymidine kinase. Acyclovir is most active against HSV; activity against VZV also is substantial but approximately ten-fold less. Epstein Barr virus (EBV) is only moderately susceptible to acyclovir

because EBV has minimal thymidine kinase activity. Activity against CMV is poor because CMV does not have a unique thymidine kinase, and CMV DNA polymerase is poorly inhibited by acyclovir triphosphate. Acyclovir is the most frequently prescribed antiviral agent. It has been available for clinical use for over two decades and has demonstrated remarkable safety and efficacy against mild to severe infections caused by HSV and VZV in both normal and immunocompromised patients.

Mechanism of action and pharmacokinetics

Acyclovir is a deoxyguanosine analogue with an acyclic side chain that lacks the 3′-hydoxyl group of natural nucleosides (Wagstaff *et al.*, 1994). Following preferential uptake by infected cells, acyclovir is monophosphorylated by virus-encoded thymidine kinase; host cell thymidine kinase is approximately 1 millionfold less capable of converting acyclovir to its monophosphate derivative. Subsequent diphosphorylation and triphosphorylation are catalyzed by host cell enzymes, resulting in acyclovir triphosphate concentrations that are 40 to 100 times higher in HSV-infected cells than in uninfected cells (Elion, 1982). Acyclovir triphosphate prevents viral DNA synthesis by inhibiting the viral DNA polymerase. In vitro, acyclovir triphosphate competes with deoxyguanosine triphosphate as a substrate for viral DNA polymerase. Because acyclovir triphosphate lacks the 3'-hydroxyl group required to elongate the DNA chain, the growing chain of DNA is terminated. In the presence of the deoxynucleoside triphosphate complementary to the next template position, the viral DNA polymerase is functionally inactivated (Reardon and Spector, 1989). In addition, acyclovir triphosphate is a much better substrate for the viral polymerase than for cellular DNA polymerase α, resulting in little incorporation of acyclovir into cellular DNA.

The oral bioavailability of acyclovir is poor, with only 15%–30% of the oral formulations being absorbed. Following a 200 mg dose, a peak concentration of about 0.5 μg/ml is attained at approximately 1.5 to 2.5 hours (Wagstaff *et al.*, 1994). Higher doses of acyclovir result in higher serum concentrations. Food does not substantially alter extent of absorption. After intravenous doses of 2.5 to 15 mg/kg, steady-state concentrations of acyclovir range from 6.7 to 20.6 μg/ml. Acyclovir is widely distributed; high concentrations are attained in kidneys, lung, liver, heart, and skin vesicles; concentrations in the cerebrospinal fluid (CSF) are about 50% of those in the plasma (Wagstaff *et al.*, 1994). Acyclovir crosses the placenta and accumulates in breast milk. Protein binding ranges from 9% to 33% and less than 20% of drug is metabolized to biologically inactive metabolites.

In the absence of compromised renal function, the half-life of acyclovir is 2 to 3 hours in older children and adults and 2.5 to 5 hours in neonates with normal creatinine clearance. More than 60% of administered drug is excreted in the urine (Wagstaff *et al.*, 1994). Elimination is prolonged in patients with renal dysfunction; the half-life is approximately 20 hours in persons with end-stage renal disease, necessitating dose modifications for those with creatinine clearance less than 50 ml/min per 1.73 m^2 (Laskin *et al.*, 1982). Acyclovir is effectively removed by hemodialysis but not by continuous ambulatory peritoneal dialysis (Krasny *et al.*, 1982).

Antiviral therapy

Clinical efficacy in HSV-1 and HSV-2 infections

Genital herpes

For the treatment of first episode genital herpes, the dose of oral acyclovir is 200 mg orally five times per day, or 400 mg orally three times per day (Table 64.1). Neither higher doses of oral acyclovir nor the addition of topical acyclovir provide added benefit (Wald *et al.*, 1994). Duration of therapy in first episode disease is 7–10 days (Anonymous, 2002). Acyclovir therapy for the treatment of first episode genital herpes reduces the duration of viral shedding by about a week, time to healing of lesions by approximately four days, and time to complete resolution of signs and symptoms by approximately two days (Bryson *et al.*, 1983; Mertz *et al.*, 1984) (Table 64.2).

For the episodic treatment of recurrent genital herpes, dosing options for acyclovir include 200 mg orally five times per day, or 800 mg orally two times per day, administered for 5 days (Anonymous, 2002) (Table 64.1). Topical acyclovir provides no clinical benefit in the episodic management of recurrences and is not recommended (Corey *et al.*, 1982; Luby *et al.*, 1984). A recent study indicates that 2 days of oral acyclovir therapy (800 mg three times per day) is also efficacious in the episodic treatment of genital HSV recurrences (Wald *et al.*, 2002). When started within 24 hours of the onset of a genital herpes recurrence, oral acyclovir reduces the duration of viral shedding by approximately two days, time to healing by just over a day, and time to complete resolution of signs and symptoms by approximately a day (Tyring *et al.*, 1998) (Table 64.2). Episodic treatment does not reduce the length of time to subsequent recurrence (Nilsen *et al.*, 1982; Reichman *et al.*, 1984; Ruhnek-Forsbeck *et al.*, 1985).

In addition to the treatment of an active genital herpes infection, acyclovir has been effectively used to prevent recurrences of genital herpes. The most frequent indication for suppressive acyclovir therapy is in patients with frequently recurrent genital infections, in whom chronic

Table 64.1. Therapeutic management of genital herpes[a]

	First clinical episode (treat orally for 7–10 days 6[b])	Episodic recurrent infection[e] (treat orally for 5 days)	Oral suppressive therapy	Episodic recurrent infection in HIV-infected persons (treat orally for 5–10 days)	Oral suppressive therapy in HIV-infected persons	Advantages	Disadvantages[f]
Acyclovir	200 mg 5×/day *or* 400 mg 3×/day	200 mg 5×/day *or* 800 mg 2×/day	400 mg 2×/day	200 mg 5×/day *or* 400 mg 3×/day	400–800 mg 2×/day *or* 3×/day	Less expensive Smaller tablets Liquid formulation available	Less convenient dosing regimens
Valaciclovir	1000 mg/ 2×/day	500 mg 2×/day[c] *or* 1000 mg 1×/day	500 mg 1×/day[d] *or* 1000 mg 1×/day	1000 mg 2×/day	500 mg 2×/day	More convenient dosing regimens	More expensive Larger caplet
Famciclovir	250 mg 3×/day	125 mg 2×/day	250 mg 2×/day	500 mg 2×/day	500 mg 2×/day	More convenient dosing regimens Smaller tablet	More expensive

[a] Modified from Reference (Anonymous, 2002).

[b] The range of duration of therapy relates to differences in treatment durations in the original clinical studies. If the shorter course of therapy is initially prescribed, the patient should be reevaluated toward the end of treatment and therapy should be continued if new lesions continue to form, if complications develop, or if systemic signs and symptoms have not abated.

[c] Three-day course of therapy also acceptable

[d] For patients with ≤9 recurrences/year

[e] When started within 24 hours of the recurrence

[f] Allergic and other adverse reactions to acyclovir, valaciclovir, and famciclovir are rare.

Table 64.2. Efficacies of acyclovir, valaciclovir, and famciclovir in the treatment of genital HSV disease

	First episode genital herpes	Episodic treatment of genital herpes recurrences	Suppressive therapy for genital herpes
Acyclovir	2-day decrease in time to complete resolution of signs and symptoms (vs. placebo) (Mertz *et al.*, 1984)	1.1-day decrease in time to complete resolution of signs and symptoms (vs. placebo) (Tyring *et al.*, 1998)	After 1 year of therapy, 44% of acyclovir recipients were recurrence-free, vs. 2% of placebo recipients (Mertz *et al.*, 1988b)
	4-day decrease in time to healing of lesions (vs. placebo) (Mertz *et al.*, 1984)	1.2-day decrease in time to healing of lesions (vs. placebo) (Tyring *et al.*, 1998)	After 4 months (120 days) of therapy, 71% of acyclovir recipients were recurrence-free, vs. 6% of placebo recipients (Douglas *et al.*, 1984)
	7-day decrease in duration of viral shedding (vs. placebo) (Mertz *et al.*, 1984)	2.0-day decrease in duration of viral shedding (vs. placebo) (Tyring *et al.*, 1998)	80–94% reduction in days with subclinical shedding (vs. placebo) (Wald *et al.*, 1997; Wald *et al.*, 1996)
Valaciclovir	Compared with acyclovir, no difference in time to healing of lesions, duration of symptoms, and duration of viral shedding (Fife *et al.*, 1997)	1.9-day decrease in time to complete resolution of signs and symptoms (vs. placebo) (Spruance *et al.*, 1996) 1.9-day decrease in time to healing of lesions (vs. placebo) (Spruance *et al.*, 1996) 2.0-day decrease in duration of viral shedding (vs. placebo) (Spruance *et al.*, 1996) Compared with acyclovir, no difference in time to healing of lesions, duration of symptoms, and duration of viral shedding (Bodsworth *et al.*, 1997; Tyring *et al.*, 1998)	After 16 weeks (112 days) of therapy, 69% of valaciclovir recipients were recurrence-free, vs. 9.5% of placebo recipients (Patel *et al.*, 1997) Compared with acyclovir, no difference in effectiveness (Reitano *et al.*, 1998) 81% reduction in days with subclinical shedding (vs. placebo) (Wald *et al.*, 1998) Compared with acyclovir, no difference in reduction of subclinical shedding (Wald *et al.*, 1998)
Famciclovir	Compared with acyclovir, no difference in time to healing of lesions, duration of symptoms, and duration of viral shedding (Loveless *et al.*, 1995)	0.5-day decrease in duration of signs and symptoms (vs. placebo) (Sacks *et al.*, 1996a) 1.0-day decrease in time to healing of lesions (vs. placebo) (Sacks *et al.*, 1996a) 1.6-day decrease in duration of viral shedding (vs. placebo) (Sacks *et al.*, 1996a) Compared with acyclovir, no difference in time to healing of lesions, duration of symptoms, and duration of viral shedding (Chosidow *et al.*, 2001)	After 4 months (120 days) of therapy, 78% of famciclovir recipients were recurrence-free, vs. 42% of placebo recipients (Mertz *et al.*, 1997b) 87% reduction in days with subclinical shedding (vs. placebo) (Sacks *et al.*, 1997)

suppressive acyclovir therapy reduces the frequency of recurrences by approximately 75% (Douglas *et al.*, 1984; Mertz *et al.*, 1988a; Mertz *et al.*, 1988b; Mindel *et al.*, 1988; Straus *et al.*, 1984) (Table 64.2). One quarter to one-third of patients on suppressive therapy experience no further recurrences while taking acyclovir. Daily administration of acyclovir maintains a high degree of efficacy and little toxicity, even after more than 5 years of continuous suppressive therapy (Goldberg *et al.*, 1993). Suppressive therapy reduces the frequency of asymptomatic shedding of HSV in the genital tract by more than 80% (Wald *et al.*, 1997; Wald *et al.*, 1996) (Table 64.2). The acyclovir dose when used as suppressive therapy is 400 mg administered twice daily (Table 64.1).

The historic rationale for episodic treatment of genital herpes was that, in many patients, the frequency and/or

severity of clinical recurrences made treatment of the recurrences desirable, but they were not sufficiently frequent or annoying to warrant daily suppressive therapy. Advances in recent years in our understanding of asymptomatic viral shedding have begun to shift our therapeutic management away from episodic treatment and toward suppressive therapy (Kimberlin and Rouse, 2004). The current rationale for suppressive treatment is as follows: (1) most persons with first episode genital herpes are at risk of frequent recurrences over the next few years (Benedetti *et al.*, 1994); (2) 70%–80% of patients receiving suppressive therapy remain recurrence-free at 4 months (Table 64.2), vs. 5%–10% of persons receiving placebo (Douglas *et al.*, 1984; Mertz *et al.*, 1988b; Mertz *et al.*, 1997b; Patel *et al.*, 1997; Reitano *et al.*, 1998); (3) days with subclinical (asymptomatic) shedding are reduced by 80%–95% compared with placebo (Table 64.2) (Sacks *et al.*, 1997; Wald *et al.*, 1997; Wald *et al.*, 1998; Wald *et al.*, 1996); (4) suppressive therapy reduces the risk of HSV transmission to uninfected partners (Corey *et al.*, 2004); (5) quality of life often is improved in patients with frequent recurrences who receive suppressive compared with episodic treatment (Alexander and Naisbett, 2002); and (6) suppressive therapy is safe (Douglas *et al.*, 1984; Mertz *et al.*, 1997b; Patel *et al.*, 1997; Reitano *et al.*, 1998).

HSV gingivostomatitis and recurrent herpes labialis

Treatment of primary gingivostomatitis in pediatric patients using oral acyclovir decreases time to cessation of symptoms by 30%–50%, and time to lesion healing by 20%–25% (Aoki *et al.*, 1993). Compared with patients receiving placebo, subjects treated with oral acyclovir at 600 mg/m^2 per dose administered four times per day for 10 days experienced cessation of drooling in 4 days (vs. 8 days in placebo recipients) and resolution of gum swelling in 5 days (vs. 7 days in placebo recipients). Intraoral lesions in acyclovir recipients healed at 6 days (vs. 8 days in placebo recipients), and extraoral lesions healed in 7 days (vs. 9 days in placebo recipients) (Aoki *et al.*, 1993).

Oral acyclovir has a more modest effect in the treatment of recurrent herpes labialis (Raborn *et al.*, 1988; Raborn *et al.*, 1987), and treatment of these patients should be individualized (Kimberlin and Prober, 2003). In general, therapeutic benefit is enhanced if treatment is initiated as soon as possible after onset of symptoms, preferably within 24 to 48 hours of onset of the recurrence. Among patients who start treatment in the prodrome or erythema lesion stage, acyclovir therapy (400 mg five times a day for 5 days) reduces the duration of pain by approximately one-third, and the healing time to loss of crust by approximately one-fourth (Spruance *et al.*, 1990). Topical acyclovir cream may also modestly decrease the duration of a clinical recurrence of herpes labialis by approximately half a day (approximately $4\frac{1}{2}$ days for topical acyclovir recipients, compared with approximately 5 days for placebo recipients) (Spruance *et al.*, 2002), although benefit of topical acyclovir is not conferred by acyclovir ointment, which has a polyethylene glycol base (Shaw *et al.*, 1985; Spruance *et al.*, 1984).

Prophylactic acyclovir also has been used to prevent reactivation of herpes labialis following exposure to ultraviolet radiation, facial surgery, or exposure to sun and wind while skiing (Gold and Corey, 1987; Spruance *et al.*, 1991; Spruance *et al.*, 1988). Topical acyclovir cream also is effective in preventing recurrent herpes labialis in skiers (Raborn *et al.*, 1997) and in persons with a history of frequent recurrences of herpes labialis (Gibson *et al.*, 1986). Long-term suppressive therapy reduces the number of recurrences of oral infection in those with histories of frequent recurrences (Rooney *et al.*, 1993). In one study of 400 mg of oral acyclovir administered twice daily for 4 months, clinical recurrences were reduced by more than half, and culture-confirmed recurrences were reduced by more than two-thirds (Rooney *et al.*, 1993).

Herpes simplex encephalitis (HSE)

For herpes simplex encephalitis, intravenous acyclovir should be administered at 30 mg/kg per day for 14–21 days for the treatment of HSE (Whitley *et al.*, 1986). Some experts recommend higher dosages of intravenous acyclovir be considered (45–60 mg/kg per day), although neurotoxicity can be a limiting factor in increasing the dose in larger children and adults. In untreated patients, mortality from HSE exceeds 70%, and only 2.5% of survivors return to normal neurologic function. Even with appropriate administration of antiviral therapy, substantial mortality and morbidity from HSE remain (Skoldenberg *et al.*, 1984), with 19% of patients dying and 62% of survivors having residual neurologic sequelae (Whitley *et al.*, 1986). Patients with a Glasgow coma score of less than 6, those older than 30 years, and those with encephalitis for longer than 4 days have a poorer outcome (Whitley *et al.*, 1986).

Neonatal HSV

For neonatal HSV disease, intravenous acyclovir at 60 mg/kg per day delivered in three divided daily doses is currently recommended (American Academy of Pediatrics, 2003; Kimberlin *et al.*, 2001a). The dosing interval of intravenous acyclovir may need to be increased in premature infants, based upon their creatinine clearance (Englund *et al.*, 1991). Duration of therapy is 21 days for patients with disseminated or CNS neonatal HSV disease, and 14 days for patients with HSV infection limited to the SEM (American Academy of Pediatrics, 2003). The primary apparent

Table 64.3. Mortality and morbidity outcomes among 295 infants with neonatal HSV infection, evaluated by the National Institutes of Allergy and Infectious Diseases' Collaborative Antiviral Study Group between 1974 and 1997[a]

	Treatment			
Extent of disease	Placebo (Whitley *et al.*, 1980a)	Vidarabine (Whitley *et al.*, 1991a)	Acyclovir (Whitley *et al.*, 1991a) 30 mg/kg per day	Acyclovir (Kimberlin *et al.*, 2001a) 60 mg/kg per day
Disseminated disease	***n* = 13**	***n* = 28**	***n* = 18**	***n* = 34**
Dead	11 (85%)	14 (50%)	11 (61%)	10 (29%)
Alive	2 (15%)	14 (50%)	7 (39%)	24 (71%)
Normal	1 (50%)	7 (50%)	3 (43%)	15 (63%)
Abnormal	1 (50%)	5 (36%)	2 (29%)	3 (13%)
Unknown	0 (0%)	2 (14%)	2 (29%)	6 (25%)
Central nervous system infection	***n* = 6**	***n* = 36**	***n* = 35**	***n* = 23**
Dead	3 (50%)	5 (14%)	5 (14%)	1 (4%)
Alive	3 (50%)	31 (86%)	30 (86%)	22 (96%)
Normal	1 (33%)	13 (42%)	8 (27%)	4 (18%)
Abnormal	2 (67%)	17 (55%)	20 (67%)	9 (41%)
Unknown	0 (0%)	1 (3%)	2 (7%)	9 (41%)
Skin, eye, or mouth infection	***n* = 8**	***n* = 31**	***n* = 54**	***n* = 9**
Dead	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Alive	8 (100%)	31 (100%)	54 (100%)	9 (100%)
Normal	5 (62%)	22 (71%)	45 (83%)	2 (22%)
Abnormal	3 (38%)	3 (10%)	1 (2%)	0 (0%)
Unknown	0 (0%)	6 (19%)	8 (15%)	7 (78%)

[a] Adapted from Kimberlin (2001).

toxicity associated with the use of this dose of intravenous acyclovir is neutropenia, with approximately one-fifth of patients with localized HSV disease (CNS or SEM) developing an absolute neutrophil count (ANC) of $\leq$1000/μl (Kimberlin *et al.*, 2001a). Although the neutropenia resolves either during continuation of intravenous acyclovir or following its cessation, it is prudent to monitor neutrophil counts at least twice weekly throughout the course of intravenous acyclovir therapy, with consideration being given to decreasing the dose of acyclovir or administering granulocyte colony stimulating factor (GCSF) if the ANC remains below 500/μL for a prolonged period of time (Kimberlin *et al.*, 2001a). All patients with CNS HSV involvement should have a repeat lumbar puncture at the end of intravenous acyclovir therapy to determine that the specimen is PCR-negative in a reliable laboratory, and to document the end-of-therapy CSF indices (Kimberlin *et al.*, 2001b). Those persons who remain PCR-positive should continue to receive intravenous antiviral therapy until PCR-negativity is achieved (Kimberlin *et al.*, 1996b; Kimberlin *et al.*, 2001b).

In the pre-antiviral era, 85% of patients with disseminated neonatal HSV disease died by one year of age, as did 50% of patients with CNS neonatal HSV disease (Whitley *et al.*, 1980a) (Table 64.3). Evaluations of two different doses of vidarabine and of a lower dose of acyclovir (30 mg/kg/day for 10 days) documented that both of these antiviral drugs reduce mortality to comparable degrees (Whitley *et al.*, 1991a; Whitley *et al.*, 1980a; Whitley *et al.*, 1983), with mortality rates at 1 year from disseminated disease decreasing to 54% and from CNS disease decreasing to 14% (Whitley *et al.*, 1991a) (Table 64.3). Despite its lack of therapeutic superiority, the lower dose of acyclovir quickly supplanted vidarabine as the treatment of choice for neonatal HSV disease due to its favorable safety profile and its ease of administration. Unlike acyclovir, vidarabine had to be administered over prolonged infusion times and in large volumes of fluid.

With utilization of a higher dose of acyclovir (60 mg/kg per day for 21 days), 12 month mortality is further reduced to 29% for disseminated neonatal HSV disease and to 4% for CNS HSV disease (Kimberlin *et al.*, 2001a) (Figs. 64.1 and

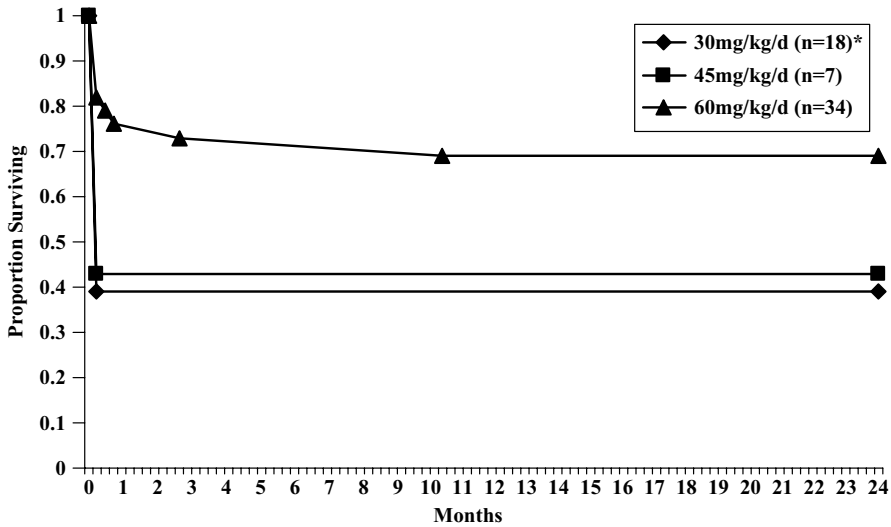

Fig. 64.1. Mortality in patients with disseminated neonatal HSV disease. (From Kimberlin *et al.*, 2001a.)

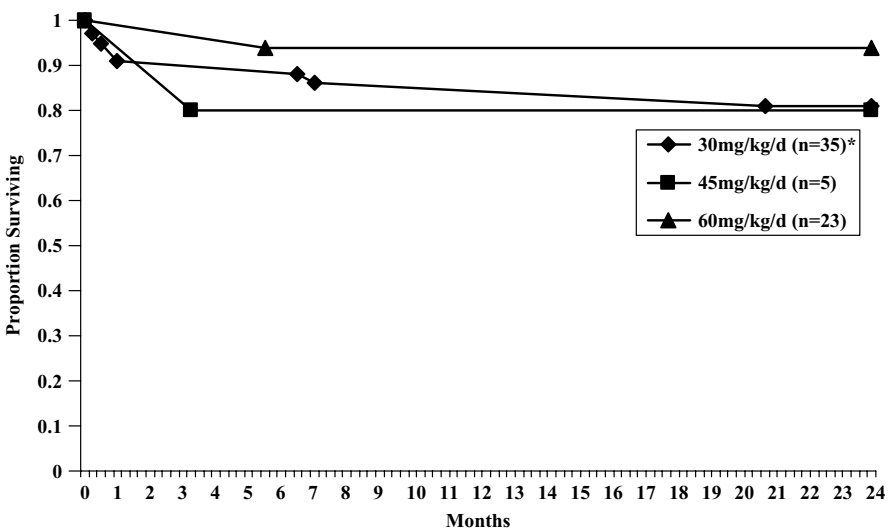

Fig. 64.2. Mortality in patients with CNS neonatal HSV disease. (From Kimberlin *et al.*, 2001a.)

64.2). Differences in mortality at 24 months among patients treated with the higher dose of acyclovir and the lower dose of acyclovir are statistically significant after stratification for disease category (CNS vs. disseminated) (Kimberlin *et al.*, 2001a). Lethargy and severe hepatitis are associated with mortality among patients with disseminated disease, as are prematurity and seizures in patients with CNS disease (Kimberlin *et al.*, 2001b).

Improvements in morbidity rates with antiviral therapies have not been as dramatic as with mortality. In the pre-antiviral era, 50% of survivors of disseminated neonatal HSV infections were developing normally at 12 months of age (Whitley *et al.*, 1980a) (Table 64.3). With utilization of the higher dose of acyclovir for 21 days, this percentage has increased to 83% (Kimberlin *et al.*, 2001a) (Fig. 64.3). In the case of CNS neonatal HSV disease, 33% of patients in the pre-antiviral era were developing normally at 12 months of age (Whitley *et al.*, 1980a) (Table 64.3), while 31% of higher dose acyclovir recipients develop normally at 12 months today (Kimberlin *et al.*, 2001a) (Fig. 64.3). While

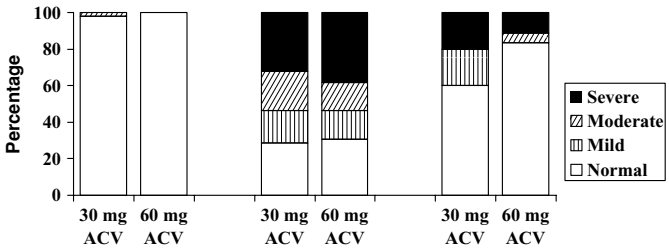

Fig. 64.3. Morbidity among patients with known outcomes after 12 months of life. (From Kimberlin *et al.*, 2001a.)

these differences are not dramatic, it is important to note that as more neonates survive neonatal HSV disease based upon the mortality data presented above, the total numbers of patients who subsequently develop normally is higher today even while the percentages of survivors with normal development are not dramatically different. Seizures at or before the time of initiation of antiviral therapy are associated with increased risk of morbidity both in patients with CNS disease and in patients with disseminated infection (Kimberlin *et al.*, 2001b).

Unlike disseminated or CNS neonatal HSV disease, morbidity following SEM disease has dramatically improved during the antiviral era. Prior to utilization of antiviral therapies, 38% of SEM patients experienced developmental difficulties at 12 months of age (Whitley *et al.*, 1980a) (Table 64.3). With vidarabine and lower dose acyclovir, these percentages were reduced to 12% and 2%, respectively (Whitley *et al.*, 1991a). In the high-dose acyclovir study, no SEM patients developed neurologic sequelae at 12 months of life (Kimberlin *et al.*, 2001a) (Fig. 64.3).

In the pre-antiviral era, 70% of neonates with disease initially limited to skin vesicles experienced progression of disease to involvement of the CNS or visceral organs (Whitley *et al.*, 1980b). It is likely that the initial reduction in morbidity among patients with SEM disease from 38% (Whitley *et al.*, 1980a) to 2–12% (Whitley *et al.*, 1991a) resulted from antiviral therapy impeding this progression to CNS or disseminated disease categories, each of which carries a higher risk of neurologic sequelae (Whitley *et al.*, 1988). The continued reduction in morbidity among patients with SEM disease seen in the recently completed high-dose acyclovir study might relate to a redefinition of what constitutes SEM vs. CNS involvement. Prior to the application of PCR technology to neonatal HSV disease, patients were classified as having SEM disease if they had no overt laboratory or clinical evidence of viral dissemination to the viscera and/or CNS. The lack of CNS involvement was manifest by no CNS symptomatology (seizures, abnormal neuroimaging studies, abnormal electroencephalograms, etc.) and normal CSF indices. As discussed above, however, PCR analysis of CSF specimens from neonates classified by these criteria as having SEM disease revealed that approximately one-quarter (7 of 29, or 24%) of these infants actually had HSV DNA present in their CSF during the acute disease course (Kimberlin *et al.*, 1996b). One of these seven patients subsequently developed significant neurologic impairment by age 12 months. Thus, it is possible that at least some of the SEM patients in the earlier studies who subsequently developed neurologic impairment actually had subclinical CNS disease, which could only be detected by means of the powerful investigative tool provided by PCR beginning in the 1990s. These data have resulted in a revised classification of CNS disease, such that a positive CSF PCR result is now sufficient to classify a patient as having CNS HSV infection.

Another possible explanation for the neurologic impairment previously experienced by some infants with SEM disease could be that, while low level viremia from the cutaneous lesions results in seeding of the CNS, initial damage to brain tissue during the acute illness does not occur either due to a host response to infection or due to antiviral therapy. Subclinical reactivation of virus within the CNS, with or without a clinical cutaneous recurrence, might then produce neurologic impairment, as has been suggested (Kimberlin, 2001; Whitley *et al.*, 1991b). Supporting this hypothesis, HSV DNA has been detected in the CSF of an SEM infant at the time of a cutaneous recurrence (Kimberlin *et al.*, 1996a). Randomized, controlled studies of long-term suppressive oral acyclovir therapy following the acute neonatal disease are currently being conducted by the NIAID Collaborative Antiviral Study Group to evaluate this hypothesis. At the current time, however, no evidence exists to suggest that suppressive oral acyclovir therapy is beneficial in preventing neurologic complications. Furthermore, almost half of infants receiving oral acyclovir in an open-label phase I/II investigation developed neutropenia while on therapy (Kimberlin *et al.*, 1996a), raising substantial questions about the safety of such a therapeutic approach outside of the strictly monitored confines of a clinical investigation.

HSV disease in the immunocompromised host

Acyclovir also is indicated for the treatment of disseminated HSV infections in otherwise normal hosts, including pregnant women, and mucocutaneous HSV infections in immunocompromised hosts (Kimberlin and Prober, 2003). Similarly, HSV infections of the lip, mouth, skin, perianal area, or genitals may be much more severe in immunocompromised patients than in normal hosts, with HSV lesions tending to be more invasive, slower to heal, and

associated with prolonged viral shedding. Intravenous acyclovir therapy is very beneficial in such patients (Wade *et al.*, 1982). Immunocompromised patients receiving acyclovir have a shorter duration of viral shedding and more rapid healing of lesions than patients receiving placebo (Meyers *et al.*, 1982). Oral acyclovir therapy is also very effective in immunocompromised patients (Shepp *et al.*, 1985).

Acyclovir prophylaxis of HSV infections is of clinical value in severely immunocompromised patients, especially those undergoing induction chemotherapy or transplantation. Intravenous or oral administration of acyclovir reduces the incidence of symptomatic HSV infection from about 70% to 5%–20% (Saral *et al.*, 1981). A sequential regimen of intravenous acyclovir followed by oral acyclovir for 3 to 6 months can virtually eliminate symptomatic HSV infections in organ transplant recipients. A variety of oral dosing regimens, ranging from 200 mg 3 times daily to 800 mg twice daily, have been used successfully. Among bone marrow transplant recipients and patients with AIDS, acyclovir-resistant HSV isolates have been identified more frequently after therapeutic acyclovir administration than during prophylaxis (Wade *et al.*, 1983).

HSV keratitis or keratoconjunctivitis

Topical therapy with acyclovir for HSV ocular infections is effective, but probably not superior to trifluridine (Hovding, 1989). Long-term suppressive therapy reduces the number of recurrences of ocular infection in those with histories of frequent recurrences (Herpetic Eye Disease Study Group, 1998, 2000).

Challenges for achieving clinical benefit, including adverse drug effects

Perhaps the most prominent challenge impacting clinical benefit of acyclovir therapy relates to the timing of drug initiation following onset of disease symptoms. In the case of life-threatening HSV disease, consideration of HSV as a possible cause of the illness is needed in order to then initiate acyclovir therapy. In the case of less severe but still consequential infections, such as primary genital herpes, the patient must present to medical attention, be correctly diagnosed, and then started on antiviral therapy as quickly as possible to achieve maximal benefit.

Acyclovir is a safe drug which is generally very well tolerated. Oral acyclovir sometimes causes mild gastrointestinal upset, rash, and headache. If it extravasates, intravenous acyclovir can cause severe inflammation, phlebitis, and sometimes a vesicular eruption leading to cutaneous necrosis at the injection site. If given by rapid intravenous infusion or to poorly hydrated patients or those with preexisting renal compromise, intravenous acyclovir can cause reversible nephrotoxicity due to the formation of acyclovir crystals precipitating in renal tubules and causing an obstructive nephropathy. Administration of acyclovir by the intravenous route occasionally is associated with rash, sweating, nausea, headache, hematuria, and hypotension. High doses of intravenous acyclovir (60 mg/kg per day) in neonates and prolonged use of oral acyclovir following neonatal disease have been associated with neutropenia (Kimberlin *et al.*, 1996a,2001b).

The most serious side effect of acyclovir is neurotoxicity, which usually occurs in subjects with compromised renal function who attain high serum concentrations of drug (Revankar *et al.*, 1995). Neurotoxicity is manifest as lethargy, confusion, hallucinations, tremors, myoclonus, seizures, extrapyramidal signs, and changes in state of consciousness, developing within the first few days of initiating therapy. These signs and symptoms usually resolve spontaneously within several days of discontinuing acyclovir.

Although acyclovir is mutagenic at high concentrations in some in vitro assays, it is not teratogenic in animals. Limited human data suggest that acyclovir use in pregnant women is not associated with congenital defects or other adverse pregnancy outcomes (Reiff-Eldridge *et al.*, 2000).

The likelihood of renal toxicity of acyclovir is increased when administered with nephrotoxic drugs such as cyclosporine or amphotericin B. Somnolence and lethargy may occur in subjects being treated with both zidovudine and acyclovir. Concomitant administration of probenicid prolongs acyclovir's half-life, whereas acyclovir can decrease the clearance and prolong the half-life of drugs such as methotrexate that are eliminated by active renal secretion.

Clinical indications

Acyclovir is licensed in the United States for the treatment of initial episodes and management of recurrent episodes of genital herpes, for the treatment of chickenpox, and for the treatment of acute herpes zoster infections. It is also indicated for the treatment of neonatal HSV disease, herpes simplex encephalitis, mucocutaneous and viscerally disseminated herpes infections in immunocompromised hosts, and the treatment of chickenpox in the normal host.

Antiviral resistance

Resistance of HSV to acyclovir has become an important clinical problem, especially among immunocompromised patients exposed to long-term therapy (Englund *et al.*, 1990). Viral resistance to acyclovir usually results from mutations in the viral TK gene although mutations in

the viral DNA polymerase gene also occur rarely. Resistant isolates can cause severe, progressive, debilitating mucosal disease and, rarely, visceral dissemination (Field and Biron, 1994; Lyall *et al.*, 1994). Isolates of HSV resistant to acyclovir also have been reported in normal hosts, most commonly in patients with frequently recurrent genital infection who have been treated with chronic acyclovir (Morfin and Thouvenot, 2003).

Famciclovir/penciclovir

Famciclovir is the inactive diacetyl ester prodrug of penciclovir, an acyclic nucleoside analogue. Following oral ingestion and systemic absorption, famciclovir is rapidly deacetylated and oxidized to form the active parent drug penciclovir.

Mechanism of action and pharmacokinetics

In cells which are infected with HSV, the viral thymidine kinase (TK) phosphorylates penciclovir to its monophosphate derivative, which in turn is converted to the active penciclovir triphosphate by cellular kinases. Penciclovir triphosphate inhibits viral DNA polymerase by competing with deoxyguanosine triphosphate for incorporation into the growing DNA strand. While penciclovir triphosphate is neither an obligate DNA chain terminator nor an inactivator of the DNA polymerase, once incorporated penciclovir triphosphate does retard the rate of subsequent nucleotide incorporation. Penciclovir is approximately 100-fold less potent than acyclovir in inhibiting herpesvirus DNA polymerase activity. By virtue of its high intracellular concentrations and long intracellular half-life (7 to 20 hours), though, it remains an effective antiviral agent.

The bioavailability of penciclovir following oral administration of famciclovir is about 70%. Peak concentrations of drug after intravenous administration of 10 mg/kg are approximately six-fold higher than those attained after oral doses of 250 mg. Food delays absorption but does not affect the final plasma drug concentration. Following oral administration, little or no famciclovir is detected in plasma or urine. The plasma half-life of penciclovir is about 2.5 hours, and almost three-quarters is recovered unchanged in the urine. Measurable penciclovir concentrations are not detectable in plasma or urine following topical administration of penciclovir cream. A 12-hour dosing interval is recommended for those with creatinine clearances between 30 and 50 ml/min per 1.73 m^2, and a 24-hour interval for those with creatinine clearances less than 30 ml/min per 1.73 m^2 (Boike *et al.*, 1994).

Antiviral therapy

Penciclovir's (and thus famciclovir's) spectrum of activity against herpesviruses is similar to that of acyclovir. In addition to HSV, penciclovir has demonstrable *in vitro* activity against VZV, EBV, and hepatitis B virus (HBV).

Clinical efficacy in HSV-1 and HSV-2 infections

Genital herpes

In the episodic treatment of genital herpes, famciclovir reduces time to healing, time to cessation of viral shedding, and durations of lesion edema, vesicles, ulcers, and crusts when compared with placebo (Sacks *et al.*, 1996b). Times to cessation of all symptoms and of moderate to severe lesion tenderness, pain, and burning are also reduced (Sacks *et al.*, 1996b). For suppression of genital HSV recurrences, famciclovir delays the time to the first recurrence of genital herpes when compared with placebo (Diaz-Mitoma *et al.*, 1998; Mertz *et al.*, 1997a). Dosing and anticipated benefits of treatment of primary and recurrent genital herpes, and of suppressive therapy, are shown in Tables 64.1 and 64.2, respectively.

Recurrent herpes labialis

Topical penciclovir (Denavir) for the treatment of recurrent herpes labialis reduces time to healing and duration of pain by about half a day (Boon *et al.*, 2000). Topical penciclovir cream decreases the time to lesion healing by approximately 1 to 2 days when compared with placebo (Boon *et al.*, 2000; Spruance *et al.*, 1997), and is equally effective as topical acyclovir cream (Lin *et al.*, 2002). Additional benefit is noted in a reduction in lesion area; faster loss of lesion-associated symptoms; and reductions in daily assessments of pain, itching, burning, and tenderness (Boon *et al.*, 2000). Faster healing and pain resolution occurs both among patients who first apply penciclovir cream in the prodrome and erythema stages and among those who start treatment in the papule and vesicle lesion stages (Spruance *et al.*, 1997). Application of medicine should begin as early as possible, preferably during the prodromal phase, and should be continued every 2 hours during waking hours for 4 days. (Diaz-Mitoma *et al.*, 1998; Mertz *et al.*, 1997a)

Challenges for achieving clinical benefit, including adverse drug effects

Famciclovir is as well tolerated as acyclovir. Complaints of nausea, diarrhea, and headache occurred in clinical trials, but at frequencies similar to those reported by placebo recipients. No clinically significant drug interactions have been reported to date, although concentrations of famciclovir among volunteers increase by about 20% in

patients receiving concomitant cimetidine or theophylline administration.

Clinical indications

Famciclovir was approved by the FDA for the treatment of acute herpes zoster in 1994, and subsequently was approved for the treatment and suppression of genital HSV disease in immunocompetent patients. Famciclovir is also approved for the treatment of recurrent mucocutaneous HSV disease in HIV-infected patients. Topical penciclovir is approved for the treatment of recurrent herpes labialis in adults.

Dosage regimens

For the episodic treatment of recurrent genital HSV disease, the dosage of famciclovir is 125 mg twice daily, administered for 5 days (Table 64.1). The recommended dose for suppression of genital HSV is 250 mg twice daily for up to 1 year (Table 64.1). Note that the lack of harmonization of treatment regimens resulted from different doses of famciclovir being studied in the clinical trials; this produced the unusual dosage recommendation of decreasing the suppression dose to treat a genital HSV recurrence. The safety and efficacy of famciclovir therapy beyond 1 year of treatment have not been established. For recurrent orolabial or genital HSV infection in HIV-infected patients, the recommended dose is 500 mg twice daily for 7 days.

Application of topical penciclovir to recurrent herpes labialis lesions should begin as early as possible, preferably during the prodromal phase, and should be continued every 2 hours during waking hours for 4 days.

Dose reduction of famciclovir is recommended for patients with compromised renal function. A 12-hour dosing interval is recommended for persons with creatinine clearances between 30 and 50 ml/min per 1.73 m^2, and a 24-hour interval for those with creatinine clearances less than 30 ml/min per 1.73 m^2 (Boike *et al.*, 1994).

The safety and efficacy of famciclovir and topical penciclovir in children have not been established. No liquid or suspension formulation exists currently.

Antiviral resistance

Because penciclovir, like acyclovir, must be activated by the viral encoded TK enzyme, TK-deficient viral strains are resistant to both acyclovir and penciclovir. Strains of HSV whose resistance to acyclovir is conferred by alteration of the TK enzyme or by DNA polymerase mutations may remain sensitive to penciclovir (Kimberlin *et al.*, 1995a).

Valaciclovir

Valaciclovir is the L-valyl ester of acyclovir that is rapidly converted to acyclovir after oral administration by first-pass metabolism in the liver (Jacobson, 1993). Licensed in 1995, it has a safety and efficacy profile similar to which of acyclovir but offers potential pharmacokinetic advantages.

Mechanism of action and pharmacokinetics

As a prodrug of acyclovir, valaciclovir has the same mechanism of action, antiviral spectrum, and resistance profiles as those of its parent drug, acyclovir. Following oral administration of valaciclovir, rapid and complete conversion to acyclovir occurs with first-pass intestinal and hepatic metabolism. The bioavailability of valaciclovir exceeds 50%, which is three to five times greater than that of acyclovir (Soul-Lawton *et al.*, 1995). Peak serum concentrations, attained about 1.5 hours after a dose, are proportional to the amount of drug administered; they range from 0.8 to 8.5 μg/ml for doses of 100 to 2000 mg (Weller *et al.*, 1993). The area under the drug concentration time curve approximates that seen after intravenous acyclovir. All other pharmacokinetic characteristics are similar to those of acyclovir (Nadal *et al.*, 2002).

Antiviral therapy

Acyclovir is most active in vitro against HSV, with activity against VZV being about tenfold less. Although EBV has only minimal thymidine kinase activity, EBV DNA polymerase is susceptible to inhibition by acyclovir triphosphate and thus EBV is moderately susceptible to acyclovir in vitro. Activity against CMV is limited by CMV's lack of a gene for thymidine kinase; furthermore, CMV DNA polymerase is poorly inhibited by acyclovir triphosphate.

Clinical efficacy in HSV-1 and HSV-2 infections

Genital herpes

Valaciclovir treatment of first-episode genital HSV is as effective as acyclovir therapy, while at the same time providing a more favorable dosing schedule compared with acyclovir (Fife *et al.*, 1997) (Tables 64.1 and 64.2). In the treatment of recurrent genital HSV, valaciclovir decreases the duration of lesions, the duration of pain, and the duration of viral shedding when compared to placebo (Spruance *et al.*, 1996). Valaciclovir also is as effective as acyclovir for the episodic treatment of recurrent genital HSV, again providing a more favorable dosing schedule compared with acyclovir (Tyring *et al.*, 1998) (Tables 64.1 and 64.2). It should be

administered for 3 to 5 days when administered as episodic treatment (Anonymous, 2002; Leone *et al.*, 2002). Valaciclovir is also effective in suppressing recurrences of genital HSV when administered as once-daily suppressive therapy (Reitano *et al.*, 1998). Valaciclovir has recently demonstrated efficacy in the suppression of recurrent herpes labialis with 500 mg once-daily (Baker *et al.*, 2000).

Recurrent herpes labialis

Valaciclovir administered at high doses for short periods of time (2 grams orally twice a day for 1 day) reduces the time to lesion healing and time to cessation of pain and/or discomfort compared to placebo, with the overall duration of the episode being decreased by approximately one day (Spruance *et al.*, 2003). However, early valaciclovir treatment does not appear to increase the likelihood that a clinical recurrence will be aborted prior to cold sore lesion development (Chosidow *et al.*, 2003; Spruance *et al.*, 2003).

Valaciclovir administered as a 500 mg dose once daily is effective in suppressing recurrences of herpes labialis, with almost two-thirds of treated patients remaining recurrence-free during four months of suppressive therapy compared with approximately one-third of placebo recipients (Baker and Eisen, 2003; Baker *et al.*, 2000).

Herpes simplex encephalitis

Herpes simplex encephalitis is managed acutely with intravenous acyclovir, as discussed above. A randomized, controlled trial of long-term suppressive oral valaciclovir therapy following the treatment of the acute HSE disease is currently being conducted by the NIAID Collaborative Antiviral Study Group. This study will determine whether subclinical reactivation of HSV within the brain contributes to the neurologic impairment experienced by many HSE survivors. At the current time, however, no evidence exists to suggest that suppressive oral valaciclovir therapy is beneficial in preventing neurologic complications.

Challenges for achieving clinical benefit, including adverse drug effects

The profiles of adverse effects and potential drug interactions observed with valaciclovir therapy are the same as those observed with acyclovir treatment. Neurotoxicity has not been reported in humans to date, although it has been observed in animal models (Jacobson, 1993). Manifestations resembling thrombotic microangiopathy have been described in patients with advanced HIV disease receiving very high doses of valaciclovir (8 grams per day), but the multitude of other medications being administered to such patients makes the establishment of a causal relationship to valaciclovir difficult (Bell *et al.*, 1997). Although causation has not been established, use of valaciclovir at such high doses should involve evaluation of potential risks and benefits.

A limited number of adverse drug interactions with acyclovir have been reported. Subjects being treated with both zidovudine and acyclovir can develop severe somnolence and lethargy. The likelihood of renal toxicity is increased when acyclovir is administered with nephrotoxic drugs such as cyclosporine and amphotericin B. Concomitant administration of probenicid decreases renal clearance of acyclovir and prolongs its half-life; conversely, acyclovir can decrease the clearance of drugs such as methotrexate that are eliminated by active renal secretion.

Clinical indications

Valaciclovir is indicated for the treatment of herpes zoster, and for the treatment or suppression of genital herpes. Although data from controlled clinical trials are limited, because of greater bioavailability, valaciclovir may be advantageous in treating infections caused by viruses relatively less sensitive to acyclovir than HSV (e.g., VZV and CMV).

Dosage regimens

Adult treatment doses for HSV-1 and HSV-2 infections are: 1) 1 gram orally twice daily for 7–10 days for first episode genital herpes (Table 64.1, 64.2) 500 mg orally twice daily for 3–5 days for episodic treatment of recurrent genital HSV disease (Table 64.1); and 64.3) 1 gram orally once daily for suppression of recurrent genital HSV (Table 64.1). Suppression of recurrent oral herpes infections has been accomplished with single daily doses of 500 mg.

Valaciclovir dosages in children are not yet established. A valaciclovir oral suspension has recently been formulated and is undergoing Phase I evaluation in infants and children.

With decreasing creatinine clearance, the dosing interval should be spread. With significant renal impairment, the dose should also be reduced in half. Acyclovir is removed during hemodialysis, and therefore an extra dose of valaciclovir should be administered following completion of hemodialysis. Supplemental doses of valaciclovir are not required following chronic ambulatory peritoneal dialysis (CAPD) and continuous arteriovenous hemofiltration/dialysis (CAVHD).

Antiviral resistance

HSV resistance to acyclovir can result from mutations in either the viral TK gene or the viral DNA polymerase gene. Although these acyclovir-resistant isolates exhibit

diminished virulence in animal models, among HIV-infected patients they can cause severe, progressive, debilitating mucosal disease and (rarely) visceral dissemination (Gateley *et al.*, 1990). Acyclovir-resistant strains of HSV also have been recovered from cancer chemotherapy patients, bone marrow and solid organ transplant recipients, children with congenital immunodeficiency syndromes, and neonates (Kimberlin *et al.*, 1996a). Although it is uncommon, genital herpes caused by acyclovir-resistant isolates has also been reported in immunocompetent hosts who usually have received chronic acyclovir therapy (Kost *et al.*, 1993).

Second-line antiviral agents for HSV-1 and HSV-2 infections cidofovir

Cidofovir was first approved for use in the United States for the therapy of AIDS-associated retinitis caused by CMV, and this remains the main indication for this antiviral agent. With a mechanism of action independent of viral TK activity, however, cidofovir can have a role in the management of HSV-1 and HSV-2 infections which are acyclovir resistant, as described below.

Mechanism of action and pharmacokinetics

Cidofovir is a novel acyclic phosphonate nucleotide analogue. In its native form, cidofovir already has a single phosphate group attached, and thus viral enzymes are not required for initial phosphorylation of drug. In this regard, it is dissimilar to the nucleoside analogues such as acyclovir and ganciclovir. Cellular kinases sequentially attach two additional phosphate groups, converting cidofovir to its active diphosphate form.

Cidofovir has a mechanism of action which is similar to other nucleoside analogues. The active cidofovir diphosphate serves as a competitive inhibitor of DNA polymerase (Ho *et al.*, 1992). While cidofovir is taken up by both virally infected and uninfected cells, the active form of the drug exhibits a 25- to 50-fold greater affinity for the viral DNA polymerase as compared to the cellular DNA polymerase, thereby selectively inhibiting viral replication (Ho *et al.*, 1992). Incorporation of cidofovir into the growing viral DNA chain results in reductions in the rate of viral DNA synthesis.

Only 2%–26% of cidofovir is absorbed after oral administration, requiring that cidofovir be administered intravenously in the clinical management of patients. The plasma half-life of cidofovir is 2.6 hours, but active intracellular metabolites of cidofovir have half-lives of 17 to 48 hours (Cundy *et al.*, 1995). Ninety percent of the drug is excreted in the urine, primarily by renal tubular secretion (Lalezari *et al.*, 1995).

Antiviral therapy

While primarily a CMV drug, cidofovir has demonstrable activity against HSV as well. Due to its unique phosphorylation requirements for activation, the drug usually maintains activity against acyclovir- and foscarnet-resistant HSV isolates (Safrin *et al.*, 1999). Although cidofovir is less potent in vitro against HSV than is acyclovir, its favorable pharmacokinetic profile increases its anti-HSV activity. Cidofovir also has demonstrated in vitro activity against varicella-zoster virus, Epstein–Barr virus, human herpesvirus-6, human herpesvirus-8, polyomaviruses, adenovirus, and human papillomavirus (HPV).

Clinical efficacy in HSV-1 and HSV-2 infections

The primary use for cidofovir at the current time is for the management of CMV retinitis in patients with acquired immunodeficiency syndrome (AIDS) (Lalezari *et al.*, 1997; Studies of Ocular Complications of AIDS Research Group in collaboration with the AIDS Clinical Trials Group, 1997). However, cidofovir has been utilized successfully in the management of disease caused by acyclovir-resistant HSV isolates (Lalezari *et al.*, 1994). Due in part to its toxicity profile (described below), cidofovir does not have a role in antiviral prophylaxis of herpesvirus infections.

The safety and efficacy of cidofovir in children have not been studied. Due to the risk of long-term carcinogenicity and reproductive toxicity, the use of cidofovir in children warrants caution.

Challenges for achieving clinical benefit, including adverse drug effects

The principle adverse event associated with systemic administration of cidofovir is nephrotoxicity. Cidofovir concentrates in renal cells in amounts 100 times greater than is seen in other tissues, producing severe proximal convoluted tubule nephrotoxicity when concomitant hydration and administration of probenicid are not employed (Cundy *et al.*, 1995; Lalezari *et al.*, 1995). When present, renal toxicity manifests as proteinuria and glycosuria. In order to decrease the potential for nephrotoxicity, aggressive intravenous prehydration and coadministration of probenecid are required with each cidofovir dose. Within 48 hours prior to delivery of each dose of cidofovir, serum creatinine and urine protein must be determined, with adjustment in dose as indicated. Due to its potential for nephrotoxicity, cidofovir should not be administered

concomitantly with other potentially nephrotoxic agents (e.g., intravenous aminoglycosides (e.g., tobramycin, gentamicin, and amikacin), amphotericin B, foscarnet, intravenous pentamidine, vancomycin, and non-steroidal anti-inflammatory agents).

Cidofovir's potential for nephrotoxicity, neutropenia, ocular hypotony, and metabolic acidosis are judged significant enough to warrant warning statements from the FDA in the package insert. Cidofovir is carcinogenic, teratogenic, and causes hypospermia in animal studies.

Clinical indications

Cidofovir is licensed for the treatment of CMV retinitis in AIDS patients. The safety and efficacy of cidofovir for the treatment of other CMV infections, including those in non-HIV-infected individuals, or of resistant HSV infections has not been established.

Dosage regimens

Due to poor oral bioavailability (2%–26%), cidofovir can only be administered intravenously or topically. The recommended induction dose of cidofovir for patients with a serum creatinine of $\leq$1.5 mg/dl, a calculated creatinine clearance $>$55 ml/min, and a urine protein $<$100 mg/dl (equivalent to $<$2 + proteinuria) is 5 mg/kg body weight administered once weekly for two consecutive weeks. The recommended maintenance dose of cidofovir is 5 mg/kg body weight administered once every 2 weeks. Aggressive intravenous prehydration and coadministration of probenecid are required with each cidofovir dose. Cidofovir must not be administered intraocularly due to the potential for ocular hypotony.

Cidofovir is contraindicated in patients with serum creatinine $>$1.5 mg/dL, calculated creatinine clearance $\leq$55 ml/min, or urine protein $\geq$100 mg/dl (equivalent to $\geq$2+ proteinuria). The maintenance dose of cidofovir must be reduced from 5 mg/kg to 3 mg/kg for an increase in serum creatinine of 0.3–0.4 mg/dl above baseline. Cidofovir therapy must be discontinued if serum creatinine increases $\geq$0.5 mg/dl above baseline.

Foscarnet

Foscarnet is an organic analogue of inorganic pyrophosphate, with the chemical name of phosphonoformic acid (PFA). As such, it is the only antiherpes drug that is not a nucleoside or nucleotide analogue. It has the potential to chelate divalent metal ions, such as calcium and magnesium, to form stable coordination compounds. It is not a first-line drug but is useful for the treatment of infections caused by resistant herpes viruses.

Mechanism of action and pharmacokinetics

Foscarnet directly inhibits DNA polymerase by blocking the pyrophosphate binding site and preventing cleavage of pyrophosphate from deoxynucleotide triphosphates (Wagstaff and Bryson, 1994). It is a non-competitive inhibitor of viral DNA polymerases or HIV reverse transcriptase, and is not incorporated into the growing viral DNA chain. It is approximately 100-fold more active against viral enzymes than host cellular enzymes.

Foscarnet is poorly absorbed after oral administration, with a bioavailability of only about 20%, thereby limiting foscarnet's delivery to the intravenous route. Maximum serum concentration attained after a dose of 60 mg/kg is approximately 500 μmol/l (Wagstaff and Bryson, 1994). Data are limited regarding tissue distribution, but CSF concentrations are about two-thirds of those in serum. Eighty percent of an administered dose of foscarnet is eliminated unchanged in the urine; half-life is 48 hours, and dosage adjustments are necessary even in the presence of minimal degrees of renal dysfunction. Hemodialysis efficiently eliminates foscarnet and therefore an extra dose of drug is recommended after a 3-hour dialysis run (MacGregor *et al.*, 1991). There are no pharmacokinetic data for foscarnet in neonates.

Antiviral therapy

Foscarnet inhibits all known human herpesviruses, including acyclovir-resistant HSV and VZV strains and most ganciclovir-resistant CMV isolates. It also is active against HIV. While the drug concentrations required for inhibition of viral replication vary markedly, they generally range from 10 to 130 μM for HSV, 100 to 300 μM for CMV, and 10 to 25 μM for HIV.

Clinical efficacy in HSV-1 and HSV-2 infections

While primarily a CMV drug, foscarnet has demonstrable activity against HSV as well, and infections caused by acyclovir-resistant strains of HSV have been successfully controlled with foscarnet (Safrin *et al.*, 1991a; Safrin *et al.*, 1991b).

The safety and efficacy of foscarnet in the pediatric population has not been established. Potential exists for deposition of foscarnet in the developing teeth and bone of children. Therefore, administration of foscarnet to pediatric patients should be undertaken only after careful evaluation

and only if the potential benefits for treatment outweigh the potential risks.

Challenges for achieving clinical benefit, including adverse drug effects

The most common adverse effects of foscarnet are nephrotoxicity and metabolic derangements. Evidence of nephrotoxicity includes azotemia, proteinuria, acute tubular necrosis, crystalluria, and interstitial nephritis (Studies of Ocular Complications of AIDS Research Group in collaboration with the AIDS Clinical Trials Group, 1992). Serum creatinine concentrations increase in up to 50% of patients, usually during the second week of therapy. Fortunately, renal function returns to normal within two to four weeks of discontinuing therapy in most affected patients. Preexisting renal disease, concurrent use of other nephrotoxic drugs, dehydration, rapid injection of large doses, or continuous intravenous infusion of drug are risk factors for developing renal dysfunction (Deray *et al.*, 1989).

Metabolic disturbances associated with foscarnet therapy include symptomatic hypo- and hypercalcemia and hypo- and hyperphosphatemia (Markham and Faulds, 1994). Hypocalcemia is due to direct chelation of ionized calcium by the drug, and patients can have such symptoms as paresthesias, tetany, seizures, and arrythmias. Metabolic disturbances can be minimized if foscarnet is administered by slow infusion, with rates not exceeding 1 mg/kg per min. Common central nervous system (CNS) symptoms associated with foscarnet therapy are headache, tremor, irritability, seizures, and hallucinations. Fever, nausea, vomiting, abnormal serum hepatic enzymes, anemia, granulocytopenia, and genital ulcerations also have been reported. The genital ulcerations appear to be associated with high urinary concentrations of drug.

Concomitant use of amphotericin B, cyclosporine, gentamicin, and other nephrotoxic drugs increases the likelihood of renal dysfunction associated with foscarnet therapy. Co-administration of pentamidine increases the risk of hypocalcemia. Anemia and neutropenia are more common when patients also are receiving zidovudine. No drug–drug interactions are known to exist with the concomitant use of foscarnet and ganciclovir.

Foscarnet's major toxicity of renal impairment is judged significant enough to warrant warning statements from the FDA in the package insert. Serum creatinine should be monitored frequently, and adequate hydration with foscarnet administration is imperative. Elevations in serum creatinine are usually, but not always, reversible following discontinuation or dose adjustment of foscarnet. Patients receiving foscarnet must also be monitored for development of mineral and electrolyte abnormalities that might result in seizures, including hypocalcemia, hypophosphatemia, hyperphosphatemia, hypomagnesemia, and hypokalemia.

Clinical indications

Foscarnet is indicated for the treatment of acyclovir-resistant mucocutaneous HSV infections in immunocompromised patients, and is the drug of choice for both HSV and VZV infections caused by acyclovir-resistant strains. Foscarnet also is indicated for the treatment of CMV retinitis in patients with AIDS.

Dosage regimens

When used for the treatment of acyclovir-resistant strains of HSV, foscarnet should be administered at 120 mg/kg per day in three divided doses. In patients with AIDS, foscarnet therapy should be initiated within 7 to 10 days of suspicion of infection caused by acyclovir-resistant HSV or VZV. Therapy should be continued until lesions have resolved.

The degree of dose reduction is proportional to reduction in creatinine clearance; when creatinine clearance is 50% of normal, the dose should be reduced by about 50%. Detailed tables of dosage adjustments are available in the foscarnet package insert.

Antiviral resistance

Foscarnet does not require activation by viral kinases, including thymidine kinase, and therefore is active *in vitro* against HSV TK-deficient mutants. Resistance occurs as a result of DNA polymerase mutations (Kimberlin *et al.*, 1995a). Strains of CMV, HSV, and VZV with three- to fivefold reduced sensitivity to foscarnet have been reported (Kimberlin *et al.*, 1995b; Safrin *et al.*, 1994; Snoeck *et al.*, 1994). These isolates may respond to therapy with acyclovir (Safrin *et al.*, 1994) or cidofovir (Snoeck *et al.*, 1994). Conversely, infections caused by acyclovir-resistant strains of HSV and VZV have been successfully controlled with foscarnet (Safrin *et al.*, 1991a; Safrin *et al.*, 1991b).

Ganciclovir

Ganciclovir is a nucleoside analogue that differs from acyclovir by having an extra hydroxymethyl group on the acyclic side chain.

Mechanism of action and pharmacokinetics

As with acyclovir and penciclovir, the first step in ganciclovir phosphorylation is carried out by a virus-encoded

enzyme, and the final steps by cellular enzymes. Ganciclovir triphosphate is a competitive inhibitor of herpesviral DNA polymerases, resulting in cessation of DNA chain elongation (Markham and Faulds, 1994). Ganciclovir triphosphate also has some activity against cellular DNA polymerases, and this potential for incorporation into cellular DNA accounts for ganciclovir's significant toxicities. Ganciclovir has similar activity to acyclovir against HSV-1, HSV-2, and VZV but, in contrast with acyclovir, its greatest activity is against CMV.

Peak serum concentrations of ganciclovir after 5 mg/kg of intravenously-administered drug range from 8 to 11 μg/ml. Concentrations of ganciclovir in the central nervous system range from 24% to 70% of those in the plasma, with brain concentrations of approximately 38% of plasma levels (Fletcher *et al.*, 1986). Most of an administered dose of ganciclovir is eliminated unchanged in the urine, with an elimination half-life of 2 to 3 hours. Intracellular ganciclovir triphosphate has a half-life of more than 24 hours.

Oral bioavailability of ganciclovir is poor, with less than 10% of drug being absorbed following oral administration (Frenkel *et al.*, 2000; Jacobson *et al.*, 1987; Markham and Faulds, 1994). Despite this, an oral dose of 1000 mg of ganciclovir produces a peak plasma concentration of 1 μg/ml. Intravitreal drug concentrations achieved during intravenous induction therapy also average 1 μg/ml, while subretinal concentrations are comparable to those achieved in plasma (Kuppermann *et al.*, 1993).

The pharmacokinetic of intravenous ganciclovir in the neonatal population are similar to those of adults (Trang *et al.*, 1993). Following intravenous administration of 6 mg/kg of ganciclovir, peak concentrations of 7.0 μg/ml are achieved. The mean elimination half-life is 2.4 hours.

Dose reduction, proportional to the degree of reduction in creatinine clearance, is necessary for persons with impaired renal function. A supplemental dose is recommended after dialysis because it is efficiently removed by hemodialysis (Swan *et al.*, 1991).

Antiviral therapy

Ganciclovir's greatest in vitro activity is against CMV, although it is also as active as acyclovir against HSV-1 and HSV-2 and almost as active against VZV.

Challenges for achieving clinical benefit, including adverse drug effects

Myelosuppression is the most common adverse effect of ganciclovir; dose-related neutropenia (less than 1000 WBC/μl) is the most consistent hematologic disturbance, with an incidence of about 40% of ganciclovir-treated patients (Markham and Faulds, 1994). Neutropenia is dose limiting in about 15% of subjects, and is reversible upon cessation of drug. Neutropenia is less frequent following oral administration of ganciclovir (Drew *et al.*, 1995). Hematopoietic growth factors may be useful in preventing or managing neutropenia. Thrombocytopenia (less than 50 000 platelets/μl) occurs in approximately 20% of treated patients, while anemia in about 2% of ganciclovir recipients. Due to its marrow suppressive effects, ganciclovir should not be administered if the absolute neutrophil count is less than 500 cells/μl or if the platelet count is less than 25 000 cells/μL.

Two to 5% of ganciclovir recipients experience headache, confusion, altered mental status, hallucinations, nightmares, anxiety, ataxia, tremors, seizures, fever, rash, and abnormal levels of serum hepatic enzymes, either singly or in some combination (Markham and Faulds, 1994). Intraocular injection of ganciclovir can cause transient increases in intraocular pressure with associated intense pain and amaurosis lasting up to 30 minutes.

Since both zidovudine and ganciclovir have the potential to cause neutropenia and anemia, some patients may not tolerate concomitant therapy with these drugs at full dosage. Renal clearance of ganciclovir decreases in the presence of probenicid. Generalized seizures have been reported in patients who received ganciclovir and imipenem-cilastatin, and these drugs should not be used concomitantly unless the potential benefits outweigh the risks.

In preclinical test systems, ganciclovir is mutagenic, carcinogenic, and teratogenic. Additionally, it causes irreversible reproductive toxicity in animal models. The use of ganciclovir in the pediatric population warrants extreme caution due to this potential for long-term carcinogenicity and reproductive toxicity. Administration of ganciclovir to pediatric patients should be undertaken only after careful evaluation and only if the potential benefits of treatment outweigh the potential risks.

Clinical indications

Ganciclovir is indicated for the treatment and prevention of CMV infections in immunocompromised patients. Its role in the treatment of HSV-1 or HSV-2 infections is limited to unique situations in which coverage of these viruses in addition to CMV is desirable.

Dosage regimens

The usual therapeutic and prophylactic dose of ganciclovir is 10 mg/kg per day, given by intravenous infusion twice a day for 2 to 3 weeks. For continued suppressive therapy to prevent relapse of infection or for long-term prophylaxis,

either of the following may be used: (1) 5 mg/kg as a single daily dose each day of the week; or (2) 6 mg/kg administered 5 days a week. Despite the absence of data, utilization of intravenous ganciclovir is largely being supplanted by oral valganciclovir in clinical practice.

Dose reduction, roughly proportional to the degree of reduction in creatinine clearance, is necessary in persons with impaired renal function (Spector *et al.*, 1995; Swan *et al.*, 1991). When creatinine clearance is between 50 and 79 ml/min per 1.73 m^2, half of the usual dose should be given every 12 hours. This same dose should be given every 24 hours if creatinine clearance is between 25 and 49 ml/min per 1.73 m^2. Twenty-five percent of the usual dose should be given every 24 hours if creatinine clearance is less than 25 ml/min per 1.73 m^2. Because ganciclovir is efficiently removed by hemodialysis, a supplemental dose is recommended after dialysis (Swan *et al.*, 1991).

Antiviral resistance

Strains of HSV that are resistant to acyclovir because of TK deficiency also are much less sensitive to ganciclovir. DNA polymerase HSV mutants that are ganciclovir-resistant have been generated in vitro but are not yet a clinical problem.

Valganciclovir

Valganciclovir was approved by the FDA in March, 2001. Because it is well absorbed after oral administration, it may represent a favorable option to intravenously-administered ganciclovir for the treatment and suppression of CMV infections in immunocompromised hosts.

Mechanism of action and pharmacokinetics

Valganciclovir is an L-valine ester prodrug of ganciclovir and as such has the same mechanism of action, antiviral spectrum, and potential for development of resistance as ganciclovir (Cocohoba and McNicholl, 2002). Valganciclovir is rapidly converted to ganciclovir, with a mean plasma half-life of about 30 minutes (Jung and Dorr, 1999). The absolute bioavailability of valganciclovir exceeds 60% and actually is enhanced by about 30% with concomitant administration of food (Brown *et al.*, 1999). The area under the curve of ganciclovir after oral administration of valganciclovir is one-third to one-half of that attained after intravenous administration of ganciclovir. Patients with impaired renal function require dosage reduction that is roughly proportional to their reduction in creatinine clearance (Cocohoba and McNicholl, 2002).

Antiviral therapy

Valganciclovir provides a more tolerable means by which ganciclovir can be delivered to the body than does intravenous ganciclovir. Studies of this drug to date have not included investigations of use for HSV infections.

Challenges for achieving clinical benefit, including adverse drug effects

Based upon data from 370 subjects participating in clinical trials, the most common side effects associated with valganciclovir therapy include diarrhea (41%), nausea (30%), neutropenia (27%), anemia (26%), and headache (22%) (Cocohoba and McNicholl, 2002).

Clinical indications

Valganciclovir has similar indications to ganciclovir. However, based upon limited controlled trials published to date, it currently is approved for the induction and maintenance therapy of CMV retinitis (Martin *et al.*, 2002).

Dosage regimens

The recommended dose of valganciclovir for induction therapy is 900 mg twice daily for 2 weeks. The recommended dose for maintenance therapy is 900 mg once daily.

Antiviral resistance

Resistance mechanisms are identical between ganciclovir and valganciclovir. Since selective pressure resulting from exposure to lower concentrations of drug appears to increase the likelihood of resistance developing among CMV isolates (Drew *et al.*, 1999), it is likely that the higher serum and tissue concentrations of ganciclovir achieved with administration of valganciclovir will produce less emergence of resistance when compared to oral ganciclovir. Whether or not this is seen in the clinical setting requires completion of Phase IV trials.

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Antiviral therapy of varicella-zoster virus infections

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Introduction

Primary infection caused by varicella-zoster virus (VZV) is manifest by varicella (chickenpox), while reactivation of latent virus causes herpes zoster (shingles). In immunocompetent children, varicella is usually not a serious disease, but can cause severe morbidity and mortality in adults and in immunocompromised individuals. Similarly, herpes zoster is associated with much greater morbidity in patients with impaired cell-mediated immune responses. In addition, herpes zoster can cause prolonged pain (postherpetic neuralgia) that can be very difficult to manage, particularly in older individuals. The outcomes of varicella and herpes zoster, especially in immunocompromised patients, have been dramatically improved by the development of safe and effective antiviral drugs with potent activity against VZV. Early drugs with modest efficacy and substantial toxicity (e.g., interferon, vidarabine, etc.) have been replaced by antiviral agents with enhanced *in vitro* activity, improved pharmacokinetic properties, and excellent safety profiles.

Diagnosis

Most experienced physicians will be able to make an accurate clinical diagnosis of chickenpox based on the distinctive appearance of the skin lesions (Fig. 65.1(a)). The clinical syndrome of a child with mild constitutional symptoms, the typical diffuse vesicular rash, and no prior history of chickenpox is strongly suggestive of the diagnosis, especially if there has been exposure to VZV within the previous two weeks. However, in countries where the incidence of varicella is dramatically declining (such as the United States), younger physicians will have fewer opportunities to see patients with chickenpox and may feel less confident with the clinical diagnosis. In addition, a variety of atypical presentations may occur in immunocompromised patients that will require laboratory confirmation. The classical dermatomal presentation of herpes zoster is also highly distinctive and readily lends itself to clinical diagnosis, although the diagnosis may be obscure initially in patients who present with dermatomal neuralgic pain prior to the onset of skin lesions (Fig. 65.1(b)).

Culture for VZV is performed by inoculating vesicular fluid onto monolayers of human fetal diploid kidney or lung cells. Unlike HSV, VZV is labile and every effort should be made to minimize the time spent in specimen transport and storage. Ideally, fluid should be aspirated from clear vesicles using a tuberculin syringe containing 0.2 ml of viral transport medium, inoculated directly into tissue culture at the bedside (or taken immediately to the laboratory), and then incubated at 36 °C in 5% CO_2 atmosphere. If no vesicles or pustules are available for aspiration, the clinician should carefully remove overlying debris or crusts from the freshest lesions available, swab the underlying ulcers, and place the swab directly into viral transport medium for rapid delivery on ice to the laboratory. Characteristic cytopathic effects are usually evident in tissue culture in 3–7 days, although cultures should be held for 14 days before they are declared negative. The culture process can be accelerated by using centrifugation cultures in shell vials. Identification of the viral isolate is confirmed by staining the monolayer with VZV-specific monoclonal antibodies. In general, viral culture for VZV is highly specific but slow, insensitive, and expensive.

Since VZV is not shed asymptomatically, demonstration of VZV virions, antigens, or nucleic acids in body fluids or tissues (other than sensory ganglia) is diagnostic of active infection. Visualization of multinucleated giant cells or herpesvirus virions in tissues by histopathology or electron microscopy does not distinguish between VZV

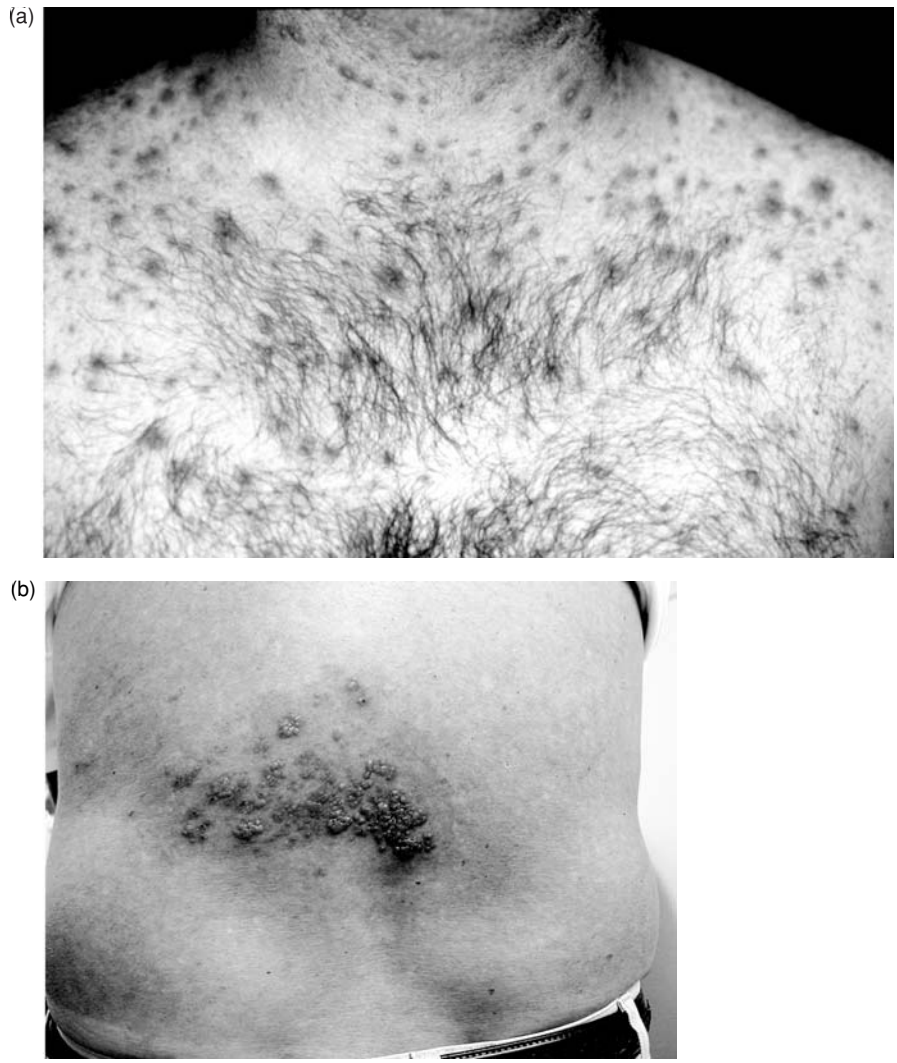

Fig. 65.1. Clinical appearance of varicella and herpes zoster. (a) Typical generalized vesicular rash of chickenpox in an adult. (b) Typical dermatomal papulo-vesicular rash of shingles in an adult. (See color plate section.)

and herpes simplex virus (HSV). Immunohistochemical staining of viral antigens can provide a more specific diagnosis. Direct fluorescent antigen (DFA) staining using fluorescein-conjugated monoclonal antibodies to detect VZV glycoproteins in infected epithelial cells is especially helpful for making a rapid diagnosis when the clinical presentation is atypical. To perform the DFA assay, epithelial cells are scraped from the base of a vesicle or ulcer with a scalpel blade, smeared on a glass slide, fixed with cold acetone, stained with fluoroescein-conjugated monoclonal antibodies, and then examined using a fluorescence microscope. By using virus-specific monoclonal antibodies, HSV can be readily distinguished from VZV, making DFA staining a much more powerful technique then a simple Tzanck preparation. DFA is also more sensitive than virus culture, especially in later stages of VZV infection when virus isolation becomes more difficult. In a population of 92 HIV-infected adults with suspected herpes zoster, DFA and viral culture were positive in 85 of 92 (92%) and 60 of 92 (65%) patients, respectively (Dahl *et al.*, 1997).

Using the polymerase chain reaction (PCR) to detect VZV nucleic acids in clinical specimens is an important diagnostic method (Stranska *et al.*, 2004). PCR overcomes the difficulties inherent in culturing labile VZV and has been used successfully to detect viral DNA in cerebrospinal fluid (CSF) from patients with VZV encephalitis

and in ocular fluids and tissues from cases of VZV retinitis. Diagnosing VZV infection of the central nervous system (CNS) can be difficult, especially when there are no concomitant cutaneous lesions. Examination of the CSF usually reveals a moderate lymphocytic pleocytosis, normal to moderately elevated protein, and normal glucose. The PCR for VZV DNA in CSF should be positive in more than 75% of cases. In one series of 34 HIV-infected patients with VZV neurologic complications, the mean CSF white blood cell count was 126/mm^3, the mean protein concentration was 230 mg/dl, and the PCR was positive for VZV in all cases (De La Blanchardiere *et al.*, 2000).

Serologic techniques can be used to determine susceptibility to VZV infection and to document rising antibody titers following varicella. Serum IgG becomes detectable several days after the onset of varicella and titers peak after 2 to 3 weeks, so routine serologic testing provides only a retrospective diagnosis. Acute infection can be confirmed by VZV-specific serum IgM titers, but antigen detection techniques are usually faster and more reliable. Patients with herpes zoster are VZV-seropositive at the time of disease onset, but most show a significant rise in antibody titer during the convalescent phase. A variety of methods have been used to detect VZV antibodies, but many laboratories have adopted an enzyme-linked immunosorbent assay (ELISA) or a latex agglutination (LA) assay for VZV serodiagnosis. The ELISA is capable of detecting IgG or IgM responses, is a reliable indicator of immune status following natural infection, and is readily automated. However, the ELISA may not be sufficiently sensitive to detect vaccine-induced immunity. The LA assay is rapid, simple, inexpensive and highly sensitive, but cannot be automated or used to detect IgM.

Drugs with activity against VZV

Acyclovir and valacyclovir

Acyclovir, an acyclic analogue of guanosine, is a selective inhibitor of VZV and HSV replication (Whitley and Gnann, 1992). The drug is converted to acyclovir monophosphate by virus-encoded thymidine kinase (TK), a reaction that does not occur to any significant extent in uninfected cells. Cellular enzymes catalyze the subsequent diphosphorylation and triphosphorylation steps which yield high concentrations of acyclovir triphosphate in VZV-infected cells. Acyclovir triphosphate inhibits viral DNA synthesis by competing with deoxyguanosine triphosphate as a substrate for viral DNA polymerase. Incorporation of acyclovir triphosphate into viral DNA results in obligate chain termination since the molecule lacks the 3-hydroxyl group required for further DNA chain elongation. Viral DNA polymerase is tightly associated with the terminated DNA chain and is functionally inactivated. Viral DNA polymerase has a much higher affinity for acyclovir triphosphate than does cellular DNA polymerase, resulting in little incorporation of acyclovir triphosphate into cellular DNA. The median inhibitory concentration of acyclovir necessary to reduce VZV plaque counts by 50% (IC50) is approximately 3 μg/ml.

After oral administration, acyclovir is slowly and incompletely absorbed with bioavailability of about 15–30%. Following oral administration of multiple doses of 200 mg or 800 mg of acyclovir, mean plasma peak concentrations at steady state are approximately 0.6 and 1.6 μg/ml, respectively. Plasma protein binding is less than 20%. Acyclovir penetrates well into most tissues, including the CNS. About 85% of an administered acyclovir dose is excreted unchanged in the urine via glomerular filtration and tubular secretion. The terminal plasma half-life of acyclovir is 2–3 hours in adults and 3–4 hours in neonates with normal renal function, but is extended to about 20 hours in anuric patients.

Valacyclovir is an orally administered prodrug of acyclovir that overcomes the problem of poor oral bioavailability and exhibits improved pharmacokinetic properties (Acosta and Fletcher, 1997). Valacyclovir, the L-valine ester of acyclovir, is well absorbed from the gastrointestinal tract via a stereospecific transporter and undergoes essentially complete first pass conversion in the gut and liver to yield acyclovir and L-valine. Using this prodrug formulation, the bioavailability of acyclovir is increased to about 54%, yielding peak plasma acyclovir concentrations that are three- to fivefold higher than those achieved with oral administration of the parent compound. Oral valacyclovir doses of 500 mg or 1000 mg produce peak plasma acyclovir concentrations of 3–4 and 5–6 μg/ml, respectively. Following administration of valacyclovir at a dose of 2 g orally four times daily, plasma acyclovir area-under-the-curve (AUC) values approximate those produced by acyclovir given intravenously at a dose of 10 mg/kg every 8 hours. Acyclovir AUC values after oral valacyclovir dosing are slightly higher in elderly individuals when compared with younger control groups, presumably due to declines in creatinine clearance associated with aging.

Acyclovir is cleared primarily by renal mechanisms so dosage modification for both acyclovir and valacyclovir are required for patients with significant renal dysfunction. The mean elimination half-life of acyclovir after a single 1 gram dose of valacyclovir is about 14 hours in patients with end-stage renal disease. Acyclovir is readily removed by hemodialysis, but not by peritoneal dialysis. No specific dosage modification for these drugs is required for

patients with hepatic insufficiency. Acyclovir and valacyclovir are not approved for use in pregnancy, but have been widely use to treat serious HSV and VZV infections in pregnant women without evidence of maternal or fetal toxicity (Reiff-Eldridge *et al.*, 2000).

Acyclovir is an extremely safe and well-tolerated drug. Local inflammation and phlebitis may occur following extravasion of intravenous acyclovir. Renal dysfunction resulting from accumulation of acyclovir crystals in the kidney has been observed following rapid intravenous infusion of large doses of acyclovir, but is uncommon and usually reversible. Acyclovir-related neurotoxicity (including agitation, hallucinations, disorientation, tremors, and mild clonous) has been reported, most often in elderly patients with underlying CNS abnormalities and renal insufficiency (Hellden *et al.*, 2003). Oral acyclovir therapy is rarely associated with either neurotoxicity or nephrotoxocity. Studies of patients receiving long-term acyclovir for chronic suppression of genital herpes have revealed no cumulative toxicity (Tyring *et al.*, 2002).

At standard doses, valacyclovir is also a very safe and well-tolerated drug (Acosta and Fletcher 1997). A syndrome of thrombotic microangiopathy (characterized by fever, microangiopathic hemolytic anemia, thrombocytopenia, and renal dysfunction) was observed in AIDS patients receiving high dose valacyclovir (8 grams per day) in a clinical trial. However, this syndrome has not been observed in immunocompetent patients receiving valacyclovir at standard doses (up to 3 grams per day). There is no contraindication to using valacyclovir at approved doses in HIV-infected patients. Clinically significant interactions between acyclovir or valacyclovir and other drugs are extremely uncommon.

Acyclovir is available in topical, oral, and intravenous formulations. The dermatologic preparation consists of 5% acyclovir in a cream or polyethylene glycol ointment base. Topical acyclovir is intended for treatment of minor mucocutaneous HSV infections and plays no role in treatment of VZV. Oral acyclovir preparations include a 200 mg capsule, 400 and 800 mg tablets, and a liquid suspension (200 mg per 5 ml). Acyclovir sodium for intravenous infusion is supplied as a sterile water-soluble powder that must be reconstituted and diluted to a concentration of 50 mg/ml. The approved dose of oral acyclovir for chickenpox is 200 mg/kg (up to a maximum of 800 mg) 4–5 times daily for 5 days. Adults with herpes zoster can be treated with oral acyclovir at a dose of 800 mg five times daily. The recommended dose of intravenous acyclovir for VZV infections is 10 mg/kg every 8 hours, although higher doses (12–15 mg/kg) are sometimes used for life-threatening infections, especially in immunocompromised patients. Dosage reduction is required in patients with renal insufficiency. Valacyclovir is available as 500 mg and 1000 mg tablets. The recommended dose for immunocompetent adults with varicella or herpes zoster is 1000 mg three times daily for 7 days. Because a suspension formulation of valacyclovir is not available, clinical experience with this drug in children with chickenpox is limited.

Penciclovir and famciclovir

Penciclovir is an acyclic guanine derivative that resembles acyclovir in chemical structure, mechanism of action, and spectrum of antiviral activity (Perry and Wagstaff, 1995). Like acyclovir, penciclovir is first monophosphorylated by viral TK, then further modified to the triphosphate form by cellular enzymes. Penciclovir triphosphate blocks viral DNA synthesis through competitive inhibition of viral DNA polymerase. Unlike acyclovir triphosphate, penciclovir triphosphate is not an obligate chain terminator and can be incorporated into the extending DNA chain. Intracellular concentrations of penciclovir triphosphate are higher then those seen with acyclovir triphosphate. In VZV infected cells, the half-life values for penciclovir triphosphate and acyclovir triphosphate are 7 hours and 1 hour, respectively. However, this potential advantage is offset by the lower affinity of penciclovir triphosphate for viral DNA polymerase. The median IC50 of penciclovir for VZV in MRC-5 cells is 4.0 μg/ml. Because penciclovir is very poorly absorbed, famciclovir (the diacetyl ester of 6-deoxy-penciclovir) was developed as the oral formulation. The first acetyl side chain of famciclovir is cleaved by esterases found in the intestinal wall and the second acetyl group is removed on first pass through the liver. Oxidation catalyzed by aldehyde oxidase occurs at the six position, yielding penciclovir.

When administered as the famciclovir prodrug, the bioavailability of penciclovir is about 77%. Following a single oral dose of 250 mg or 500 mg of famciclovir, peak plasma penciclovir concentrations of 1.9 and 3.5 μg/ml are achieved at 1 hour. The pharmacokinetics of penciclovir are linear and dose dependent over a famciclovir dosing range of 125–750 mg. Penciclovir is not metabolized, but is eliminated unchanged in urine, with an elimination half-life of about 2 hours after intravenous administration. Penciclovir for intravenous administration has not been commercially marketed. Famciclovir is available as 125 mg, 250 mg, and 500 mg tablets. In the United States, the recommended dose of famciclovir for uncomplicated herpes zoster is 500 mg three times daily. Famciclovir doses of 250 mg three times daily and 750 mg once daily are approved for treatment of shingles in some countries and appear to be comparable with respect to cutaneous healing of herpes

zoster (Shafran *et al.*, 2004). Adjustment of the famciclovir dose is required in patients with creatinine clearance of <60 ml/min. The adverse effects most frequently reported by patients participating in clinical trials of famciclovir were headache and nausea, although these symptoms did not differ significantly between famciclovir and placebo recipients.

Other drugs

Brivudin

Brivudin (bromovinyl deoxyuridine) is a highly potent thymidine nucleoside analogue with selective activity against HSV-1 and VZV (Keam *et al.*, 2004). The mechanism of action of brivudin appears to be inhibition of the viral DNA polymerase. The drug is well-absorbed after oral administration and has a favorable pharmacokinetic profile which permits once-daily dosing. Brivudin is generally well-tolerated; nausea is the most frequently reported adverse event. Because of concerns about the safety profile of the drug, commercial development of brivudin was halted in the United States. The drug is available in several countries as a 125 mg tablet and as a 0.1% ointment for ophthalmologic use.

Foscarnet

Foscarnet (phosphonoformic acid) is a pyrophosphate analogue that functions as an inhibitor of viral DNA polymerase by blocking the pyrophosphate binding site (Wagstaff and Bryson, 1994). Unlike the nucleoside analogues discussed above, foscarnet does not require intracellular activation by TK, therefore, TK-deficient HSV and VZV isolates that are resistant to acyclovir and related drugs remain susceptible to foscarnet. Foscarnet is administered only by the intravenous route and 80%–90% of an administrated dose is excreted unchanged in the urine. The appropriate dose of foscarnet for treatment of acyclovir-resistant VZV infections has not been assessed systematically, but doses ranging from 40 mg/kg every 8 hours to 100 mg/kg every 12 hours have been used successfully. The most important adverse effect associated with foscarnet therapy is nephrotoxicity. Dose limiting renal toxicity was noted in 15%–20% of patients treated with foscarnet for CMV retinitis. Loading the patient with intravenous saline prior to foscarnet infusion can help reduce the risk of nephrotoxicity. Foscarnet can also induce a variety of electrolyte and metabolic abnormalities, most notably hypocalcemia. Foscarnet-induced electrolyte disturbances can predispose the patient to cardiac arrhythmias, tetany, altered mental status, or seizures. To avoid serious adverse effects that can result from bolus infusion, foscarnet must be administered with an infusion pump over a duration of at least one hour. Serum creatinine levels should be checked at least three times weekly in patients receiving foscarnet and the dosage adjusted according to the manufacturer's guidelines.

Vidarabine

Vidarabine (adenine arabinoside) was the first intravenous antiviral drug accepted for widespread clinical use and was shown to be effective for VZV infections in immunocompromised patients. Vidarabine has now been replaced by more effective and less toxic antiviral drugs.

Interferon

Administration of alpha-interferon to immunocompromised patients with herpes zoster reduces the risk of viral dissemination, but has little impact on dermatomal rash healing or pain. Interferon therapy was associated with significant adverse events and has been supplanted by more specific antiviral drugs.

Clinical indications for therapy

Varicella

Children

In healthy children, varicella is associated with low rates of morbidity and mortality. For most children, supportive care alone is sufficient. Astringent soaks, antipruritics, and antipyretics (preferably acetaminophen) improve comfort. Trimming the fingernails closely helps prevent bacterial superinfections caused by scratching. If bacterial cellulitis (especially caused by group A streptococcus) develops, antibiotics may be required.

Oral acyclovir has been evaluated for treatment of uncomplicated varicella in immunocompetent children (Balfour *et al.*, 1990; Dunkle *et al.*, 1991). Acyclovir therapy, initiated within 24 hours of the onset of rash, resulted in shorter duration of fever, fewer skin lesions, and accelerated lesion healing. Overall, oral acyclovir was well tolerated and reduced the duration of symptomatic illness by about 24 hours. The populations studied in these clinical trials were not significantly large to assess the impact of acyclovir therapy on the incidence of varicella complications. Unlike acyclovir, valacyclovir and famciclovir are not available as suspension formulations and have not been evaluated extensively for treatment of varicella in small children. Some pediatricians still view antiviral therapy as optional for otherwise healthy children with chickenpox. Since the introduction of the varicella vaccine in the United

Table 65.1. Antiviral therapy for VZV infections

	Drug	Dose[a]	Major toxicities
Immunocompetent patients			
Varicella	Acyclovir	20 mg/kg (800 mg max.) po 5 times daily × 5 d. In adults, famciclovir and valacyclovir will also likely be effective.	None; minor nausea or headache
Herpes zoster	Acyclovir	800 mg po 5 times daily × 7–10 d	As above
	Valacyclovir	1000 mg po every 8 h × 7 d	None; minor nausea or headache
	Famciclovir	500 mg po every 8 h × 7 d	None; minor nausea or headache
	Brivudin[b]	125 mg po once daily × 7 d	Potentially lethal interaction with fluoropyrimidines (e.g., 5-fluorouracil)
Immunocompromised patients			
Varicella	Acyclovir	10–15 mg/kg (or 500 mg /m^2) intravenously every 8 h for ≥7 d	Nephrotoxicity (rare); CNS disturbances (rare)
Herpes zoster	Acyclovir	IV therapy (as above). Mild to moderately immunocompromised patients (including most AIDS patients) can be treated with oral therapy.	As above
Disseminated VZV syndromes (e.g., encephalitis, pneumonitis)	Acyclovir	IV therapy (as above)	As above
Infection caused by acyclovir-resistant VZV	Foscarnet	60–90 mg/kg intravenously every 12 h until healed (≥10 d)	Nephrotoxicity (common): electrolyte disturbances (common), seizures, arrhythmias, anemia, genital ulcers

[a] Doses given are for adults with normal renal function.
[b] Not licensed in the United States.

States in 1995, the incidence of chickenpox has declined dramatically, reducing the need for antiviral options in this population.

Adults

Immunocompetent adolescents and adults with varicella can be seriously ill, with high fever, hundreds of cutaneous lesions, incapacitating constitutional symptoms, and a higher risk of complications (especially pneumonitis). Since they are likely to miss at least seven days of school or work, interventions that will reduce the duration of the acute illness are warranted. In a placebo-controlled trial of therapy for 148 adults with varicella, acyclovir (800 mg orally five times daily) was shown to reduce the duration of new lesion formation, reduce the maximum number of lesions, accelerate cutaneous healing, and shorten the duration of fever (Wallace *et al.*, 1992). Similarly, a study of acyclovir treatment in otherwise healthy adolescents demonstrated shorter duration of new lesion formation and of constitutional symptoms, including fever (Balfour *et al.*, 1992). In these studies, the benefit of acyclovir therapy was minimal when treatment was initiated later than 24 hours after rash onset. Overall, acyclovir reduced the duration of illness by about two days. Valacyclovir and famciclovir are also likely to be effective in this setting, but data from controlled clinical trials are lacking. While antiviral therapy is considered optional for healthy children with varicella, the higher potential for morbidity clearly favors treatment in adolescents and adults (Table 65.1). Available data are insufficient to determine whether acyclovir therapy reduces the risk of complications such as pneumonitis or encephalitis.

In immunocompetent patients, visceral dissemination of varicella most often involves the CNS (presenting as cerebellar ataxia, encephalitis, transverse myelitis, or stroke syndromes) or the lungs (viral pneumonitis) (Gnann, 2002). No controlled studies of antiviral therapy for these complications of varicella have been performed. However, information derived from clinical experience and case reports suggests that intravenous acyclovir (10–15 mg/kg every 8 hours) may be beneficial (Haake *et al.*, 1990; Wilkins *et al.*, 1998) (Table 65.1).

The decision whether to initiate antiviral therapy in a patient with chickenpox will hinge on the patients age, underlying medical conditions, and the risk of complications (Arvin, 2002). In general, young children (under age

12 years) are at lower risk for complications than are adolescents or adults. An exception may be secondary pediatric cases in a household, who tend to have more severe disease than the index case. Benefits of antiviral therapy are minimal for healthy children presenting with greater than 24 hours of illness. Because of the greater risk of complications, antiviral therapy is appropriate for adolescents and adults with chickenpox, probably even for those presenting 48–72 hours into the course of illness. Immunocompromised patients with varicella are at significant risk for viral dissemination and visceral involvement and should always receive antiviral therapy.

Pregnant women

Although based more on case reports than on prospectively acquired data, the evidence that varicella in pregnancy is associated with enhanced morbidity is compelling (Nathwani *et al.*, 1998). Women who contract varicella while pregnant have an estimated 10% risk for developing severe VZV pneumonitis. Aggressive antiviral therapy is recommended for a pregnant woman with varicella who develops any evidence of pulmonary involvement, including cough, shortness of breath, or abnormal chest radiograph. Data from clinical trials are lacking, but several case series have reported clinical improvement in pregnant women with varicella pneumonia who were treated with intravenous acyclovir. Although acyclovir is not approved for use during pregnancy for any indication, no fetal toxicity attributable to acyclovir has been demonstrated and the risk-benefit ratio clearly supports the use of acyclovir in the setting of maternal varicella pneumonia (Reiff-Eldridge *et al.*, 2000). Many experts favor antiviral therapy (with acyclovir or valacyclovir) for all pregnant women with chickenpox in an effort to reduce maternal morbidity. No data are available to indicate whether treating the mother will alter the risk of the rare fetal varicella syndrome (Harger *et al.*, 2002).

Immunocompromised patients

The availability of safe and effective antiviral drugs has greatly reduced the high mortality rate previously associated with varicella in immunocompromised patients. Populations at high risk include organ transplant recipients, patients with cancer (especially hematologic malignancies), and other patients receiving immunosuppressive medications (including corticosteroids). Because of the high frequency of visceral involvement in immunocompromised children (or adults) with chickenpox, antiviral therapy is mandatory (Nyerges *et al.*, 1988). A small placebo-controlled trial of intravenous acyclovir in immunocompromised children with varicella demonstrated a dramatic reduction in the frequency of VZV pneumonitis from 27% to 0% (Prober *et al.*, 1982). Therapy with intravenous acyclovir (10 mg/kg or 500 mg/m^2 every 8 hours for 7–10 days) should be initiated at the first sign of infection. A switch to oral antiviral therapy (acyclovir, valacyclovir, or famciclovir) can be considered when the patient is afebrile and new lesion formation has ceased. When feasible, the dosage of immunosuppressive medications should be temporarily reduced in immunosuppressed patients with varicella. Despite the lack of data from large-scale controlled trials, the safety and efficacy of intravenous acyclovir have led to its acceptance as the drug of choice for varicella in severely immunocompromised patients (Table 65.1). Oral antiviral therapy may be efficacious in modestly immunocompromised patients (e.g., those with solid tumor malignancies or a low-dose corticosteroids), but prospectively acquired data are limited. In a retrospective review of 14 pediatric heart transplant recipients with varicella, half received intravenous acyclovir and half received oral valacyclovir; all patients recovered without serious complications (Dodd *et al.*, 2001).

Patients with HIV infection

Varicella does not appear to be unusually severe in most HIV-seropositive children, although some investigators have reported a longer duration of new lesion formation and higher median lesion counts. A variety of varicella complications in HIV-infected children have been reported (including DIC, pneumonitis, hepatitis, and encephalitis), although reliable incidence figures are not available. Deaths attributable to chickenpox in children with HIV infection are rare and are usually due to pneumonitis. No controlled prospective studies of antiviral therapy for chickenpox in HIV-infected children have been reported, so recommendations must be derived from anecdotal experience. Most clinicians prescribe oral antiviral therapy, reserving intravenous acyclovir for patients with unusually severe or complicated infections (Gershon *et al.*, 1997).

Herpes zoster

Immunocompetent adults

The goals of therapy for herpes zoster in immunocompetent adults are to accelerate the events of cutaneous healing, reduce the severity of acute neuritis, and most importantly, to reduce the incidence, severity, and duration of chronic pain (Gnann and Whitley, 2002). Even without antiviral therapy, the cutaneous lesions of herpes zoster almost always resolve within a month. However, chronic pain (postherpetic neuralgia) can persist for months or even years and is the most significant manifestation of herpes zoster in the normal host (Johnson, 2002). Three oral

antiviral drugs are currently approved in the United States for treatment of herpes zoster. Acyclovir, valacyclovir, and famciclovir have been demonstrated to reduce the duration of viral shedding, promote resolution of skin lesions, and limit the duration of pain when antiviral therapy is initiated within 72 hours of lesion onset (Table 65.1).

In placebo-controlled trials, oral acyclovir (800 mg five times daily for 7 days) was shown to accelerate cutaneous healing and to reduce the severity of acute neuritis in immunocompetent adults with herpes zoster (McKendrick *et al.*, 1986; Huff *et al.*, 1988; Wood *et al.*, 1988; Morton and Thomson, 1989). Overall, acyclovir therapy reduced the duration of new vesicle formation by about 1.5 days and the time to 50% lesion healing by about 2.5 days. These clinical trials with acyclovir showed variable benefit for reduction of the frequency and duration of postherpetic neuralgia (PHN), partially due to limitations in study design and population size. Data from these studies were reexamined in another analysis which demonstrated that acyclovir was significantly superior to placebo for reducing the duration of "zoster associated pain," defined as the continuum of pain measured from initial onset until final resolution (Wood *et al.*, 1996). Among patients ≥50 years of age, the median time to resolution of pain was 41 days and 101 days and the proportion with persistent pain at 6 months was 15% and 35% in the acyclovir and placebo treatment groups, respectively. Intravenous acyclovir is also effective in this setting, but is impractical for outpatient management of most patients with shingles. Extending oral acyclovir therapy beyond 7 days does not produce any additional benefit (Wood *et al.*, 1994).

Valacyclovir (1000 mg three times daily for 7 days) was compared with oral acyclovir in a study of 1141 immunocompromised patients over 50 years of age with herpes zoster (Beutner *et al.*, 1995). When initiated within 72 hours of lesion onset, the two drugs were equivalent for accelerating the events of cutaneous healing, but valacyclovir was superior to acyclovir in shortening the median time to resolution of zoster associated pain (38 days vs. 51 days; $P = 0.001$). The proportion of patients still experiencing pain at six months was 25.7% in the acyclovir treatment group and 19.3% in the valacyclovir groups ($P = 0.02$). Extending valacyclovir therapy to 14 days did not result in any additional benefit.

In a controlled trial conducted in 419 immunocompetent patients presenting within 24 hours of lesion onset, famciclovir (500 mg three times daily) was significantly superior to placebo in reducing the duration of viral shedding, limiting the duration of lesion formation, and accelerating the events of cutaenous healing (Tyring *et al.*, 1995). In a subset of shingles patients ≥50 years of age who had persistent pain after skin healing ($n = 170$), the median duration of PHN was reduced from 163 days to 63 days ($P = 0.004$) in the placebo and famciclovir treatment Groups, respectively. In a study comparing famciclovir and acyclovir, the two drugs were shown to have similar efficacy for herpes zoster (Degreef and the Famciclovir Herpes Zoster Study Group, 1994).

Valacyclovir and famciclovir were compared for treatment of herpes zoster in immunocompetent patients in a randomized clinical trial. In this population of 597 patients ≥50 years of age enrolled within 72 hours of rash onset, the two drugs were shown to be therapeutically equivalent, both in terms of cutaneous healing and pain resolution (Tyring *et al.*, 2000). At six months after onset of shingles, 19% of patients in each treatment group still reported pain. Acyclovir, valacyclovir, and famciclovir are all well tolerated and appear to be approximately comparable in clinical efficacy for managing herpes zoster in the immunocompetent host. Because their improved pharmacokinetic properties allow simpler dosing regimens, valacyclovir and famciclovir are preferred over acyclovir for this indication. Comparative drug cost is also a legitimate variable in selecting an antiviral drug for treatment of herpes zoster.

Brivudin (125 mg once daily × 7 days) was compared with acyclovir (800 mg 5 times daily × 7 days) in a study of 1227 immunocompetent adults with herpes zoster. Brivudin was judged to be superior to acyclovir for reducing the time to cessation of new vesicle formation and equivalent to acyclovir in terms of cutaneous healing and acute pain alleviation (Wassilew and Wutzler, 2003a,b). In a follow-up survey of subjects ≥50 years old, the incidence of PHN was lower in brivudin recipients (32.7%) than in acyclovir recipients (43.5%) (Wassilew and Wutzler, 2003a,b). Brivudin is commercially available in several European Union countries, but has not been approved in the United Kingdom or the United States because of concerns about potential drug-related toxicities (Gross *et al.*, 2003).

Certain characteristics have been defined which identify immunocompetent patients at highest risk for complications of shingles and thus most likely to benefit from antiviral therapy. Careful studies have clearly showed that older age, greater skin surface area involved with herpes zoster, and severity of pain at time of clinical presentation are all predictors of more severe and long lasting pain (Wood *et al.*, 1996; Dworkin *et al.*, 1998; Harrison *et al.*, 1999; Whitley *et al.*, 1999; Nagasako *et al.*, 2002). Patients meeting these criteria should be targeted for therapy with antiviral drugs and potent analgesics. Conversely, patients under 50 years of age are at lower risk for severe or prolonged pain and an argument could be made that antiviral therapy in

this group is optional. Available efficacy data from published studies relate to patients who present within 72 hours of lesion onset, although patients frequently present for medical care beyond that window (Wood *et al.*, 1998). The presence of new vesicles correlates with recent viral replication and may be a marker for patients who would benefit from antiviral therapy, even beyond 72 hours. In addition, patients presenting with the high-risk characteristics cited above should be considered for antiviral treatment, even when presenting beyond 72 hours after lesion onset. However, patients whose lesions that have all begun to crust are unlikely to derive benefit from antiviral therapy.

Adding corticosteroids to antiviral therapy in patients with acute herpes zoster has been suggested as a way to reduce pain. A study conducted in the United Kingdom compared acyclovir with and without prednisolone in 400 immunocompetent patients over 18 years of age (Wood *et al.*, 1994). Another clinical trial, conducted in the United States, enrolled 208 patients over 50 years of age into a four-armed study (acyclovir plus placebo, prednisone plus placebo, acyclovir plus prednisone, placebo plus placebo) (Whitley *et al.*, 1996). Both studies targeted patients within 72 hours of the appearance of lesions. Both of these studies demonstrated that corticosteroid therapy led to a reduction of pain during the acute phase of herpes zoster, but neither showed any reduction in the risk of postherpetic neuralgia (Wood *et al.*, 1994; Whitley *et al.*, 1996). Addition of corticosteroids to antiviral therapy for treatment of herpes zoster in selected older adults may result in improvements in quality of life measurements such as reduction in time to uninterrupted sleep, reduction in time to return to usual activities, and reduction in analgesic use (Whitley *et al.*, 1996). In the American trial cited above, prednisone was given for three weeks (60 mg daily for 7 days, 30 mg daily for 7 days, and 15 mg daily for 7 days), although it is possible that shorter courses of prednisone are also effective. Corticosteroid therapy can have significant adverse effects and should not be used in patients at risk for steroid toxicity (e.g., patients with diabetes mellitus, gastritis, etc.). Although only the combination of corticosteroids plus acyclovir has been studied, combination therapy using valacyclovir or famciclovir is assumed to be equally effective. Use of corticosteroids for herpes zoster without concomitant antiviral therapy is not recommended. Furthermore, use of corticosteroids in immunocompromised patients with herpes zoster has not been evaluated and is not recommended.

Symptomatic measures should be suggested to keep the patient with herpes zoster more comfortable. Patients should keep the cutaneous lesions clean and dry to reduce the risk of bacterial superinfection. Patients may wash the skin lesions with soap and water in the shower and then carefully pat the skin dry with a clean towel. Some patients find warm or cool astringent soaks (e.g., Domeboro® solution) to be soothing. A sterile non-occlusive, non-adherent dressing placed over the involved skin will protect the lesions from contact with clothing, which may be especially helpful for patients with increased skin sensitivity (i.e., allodynia). There is no role for topical creams or ointments (including topical acyclovir or penciclovir) in management of herpes zoster. The acute pain of shingles can be very severe and should not be underestimated by the clinician. The pain may be disproportionate to the rash; that is, patients with limited skin involvement can still have severe neuralgic pain. Pain is the most important symptom of herpes zoster and should be aggressively managed. In patients with severe neuralgic pain, sympathetic nerve blocks can provide rapid, but temporary relief (Opstelten *et al.*, 2004). Short-acting narcotic analgesics given on a scheduled (rather than as-needed) basis should be prescribed. Some models used to explain the pathogenesis of PHN suggests that early attenuation of acute pain will reduce the degree of nociceptive input that reaches the spinal cord neurons and prevent the initiation of central mechanisms of chronic pain, thereby reducing the risk of PHN (Dworkin *et al.*, 2000).

Medical management of established PHN is complex and often requires a multifaceted approach (Dworkin and Schmader, 2003; Johnson and Dworkin, 2003). Opioid analgesics are the mainstay of therapy during the acute phases of neuralgic pain (Table 65.2). A clinical trial with controlled-release oxycodone for patients with PHN demonstrated a significant level of pain reduction (67% of those receiving oxycodone versus 11% receiving placebo) as measured by visual analogue scale (Watson and Babul, 1998). Long-acting opioid preparations (oral or transdermal) are preferable to short-acting analgesics for management of chronic PHN. Several randomized, controlled clinical trials have shown tricyclic antidepressants (including amitriptyline, nortriptyline and desipramine) to be effective in reducing the pain of PHN, either as a single agent or in combination with other drugs (Raja *et al.*, 2002; Bowsher, 2003). Because tricyclic antidepressants are frequently associated with sedation and anticholinergic side effects, treatment should begin with a relatively low dose at bedtime, with a gradual increase in dosage as required and tolerated. Nortriptyline is as efficacious as amitriptyline for PHN, but nortriptyline is associated with fewer adverse effects in elderly patients (Watson *et al.*, 1998). In two clinical trial, the anticonvulsant gabapentin was shown to significantly reduce established PHN when

Table 65.2. Management of postherpetic neuralgia

Drug	Dosing	Comments	Adverse effects
Opioid analgesics	Varies with drug	Begin with short-acting drug at morphine oral equianalgesic dose of 5–15 mg every 4 hr. After 2 wk, convert to equianalgesic does of long-acting drug[a]	Sedation, nausea, constipation, dizziness, dependence, abuse, overdose
Gabapentin[b]	Begin with 100 mg po every 8 hr	Titrate dose up by 300 mg/d (in divided doses) to target dose of 1800–3600 mg/d, as tolerated	Somnolence, dizziness, ataxia, peripheral edema
Tricyclic antidepressants	Nortriptyline 25 mg po at bedtime	Titrate dose up to 75–150 mg/d, as necessary. Amitriptyline also effective but may cause more adverse effects in elderly patients. Desipramine is option if nortriptyline causes excess sedation	Sedation, confusion, anticholinergic effects (dry mouth, blurred vision, constipation, urinary retention)
Lidocaine (5%) patch	Apply to painful area; up to 3 patches can be used at a time for a maximum of 12 hr daily	Apply only to healed, intact skin. Patches may be cut to size. Especially helpful for allodynia. Benefit apparent within 2 weeks.	Localized skin irritation only. Systemic toxicity from cutaneous absorption of lidocaine is very rare.

[a] Options for long-acting opioid analgesics include: controlled-release morphine, controlled-release oxycodone, transdermal fentanyl, levorphanol, methadone.
[b] Pregabalin is available as an alternative to gabapentin.
(Modified with permission from Gnann and Whitley, *N. Engl. J. Med.*, 2002.)

used alone or in combination with other modalities (Rowbotham *et al.*, 1998; Rice and Maton, 2001). For treatment of PHN, physicians should initiate gabapentin at a low dose of 100 mg three times daily and escalate (in increments of 100 mg t.i.d.) as required, watching for adverse effects such as somnolence, dizziness, and ataxia. Total daily doses of 1800–3600 mg may be required (Stacey and Glanzman, 2003). Pregabalin has also been shown to be effective and well-tolerated in studies of patients with PHN and is likely to replace gabapentin for this indication (Dworkin *et al.*, 2003; Sabatowski *et al.*, 2004). The adverse effects of these medications can be additive (such as sedation due to opioid analgesics, tricyclic antidepressants, and gabapentin), especially in elderly patients (Schmader, 2001). Local transdermal administration of lidocaine via patches has been shown to significantly reduce PHN in two controlled trials (Davies and Galer, 2004). Topical treatments should only be used on intact healed skin. Topical application of capsaicin cream can provide relief of PHN for some patients, but the local stinging and burning associated with capsaicin may be intolerable for many individuals. In a controlled clinical trial of 277 patients with intractable PHN, intrathecal injection of 60 mg of methylprednisolone acetate once weekly for 4 weeks resulted in significant pain reduction, but these results require confirmation (Kotani *et al.*, 2000). There is no evidence that prolonged administration of antiviral drugs has any benefit for treatment of established PHN (Acosta and Balfour, 2001).

Herpes zoster ophthalmicus

Special emphasis should be given to patients presenting with herpes zoster involving the first division of the trigeminal nerve because of the potential for sight-threatening ocular complications. The ophthalmic division of the trigeminal nerve is the cranial nerve most frequently affected by herpes zoster. Without antiviral therapy, 50% of patients with herpes zoster ophthalmicus (HZO) will develop significant ocular complications (which can include neurotrophic keratopathy, episcleritis, iritis, epithelial or stromal keratitis, etc.) (Liesegang, 1999). Controlled prospective clinical trials clearly demonstrated that oral acyclovir therapy reduced the frequency of serious late ocular inflammatory complications of HZO from about 50%–60% to 20%–30% (Cobo *et al.*, 1986; Harding and Porter, 1991; Herbort *et al.*, 1991; Hoang-Xuan *et al.*, 1992; Beutner *et al.*, 1995). A clinical trial comparing the efficacy of valacyclovir and acyclovir for HZO demonstrated the two drugs to be comparable (Colin *et al.*, 2000). Similarly, a controlled study comparing acyclovir and famciclovir in 454 patients with HZO found that the prevalence of severe and non-severe ocular manifestations (58%) was the

same for both treatment groups (Tyring *et al.*, 2001a,b). Some experts favor intravenous acyclovir as initial therapy for patients (especially immunocompromised patients) with severe HZO. Systemic antiviral therapy has largely replaced topical antiviral preparations for treatment of the ocular complications of HZO. Systemic or topical corticosteroids may be indicated for some of the ocular inflammatory phenomena that accompany HZO (e.g., uveitis), but should only be administered under the supervision of an experienced ophthalmologist (Liesegang, 1999). Available data strongly support the routine and early use of systemic antiviral therapy in all patients with HZO in an effort to reduce the risk of ocular complications (Severson *et al.*, 2003; Zaal *et al.*, 2003).

Immunocompromised patients

Patients with disorders of cell-mediated immunity are at increased risk for development of herpes zoster. In this population, those patients with the greatest degree of immunosuppression (such as hematopoietic stem-cell transplant (HSCT) recipients or patients with lymphoproliferative malignancies) are at highest risk for VZV dissemination and visceral organ involvement. Clinical trials with intravenous acyclovir for localized or disseminated herpes zoster in immunocompromised patients clearly demonstrated that treatment resulted in more rapid virus clearance and halted disease progression (Serota *et al.*, 1982; Balfour *et al.*, 1983). Subsequent studies in HSCT recipients have demonstrated that acyclovir, in addition to promoting faster disease resolution, is highly effective at preventing VZV dissemination (Meyers *et al.*, 1984; Shepp *et al.*, 1986). Because most VZV-related fatalities result from disseminated infection, the ability to prevent dissemination has markedly reduced the herpes zoster mortality rate in immunocompromised patients. In addition, intravenous acyclovir is considered the drug of choice for treating dissemination when it occurs, although efficacy data from prospective studies are limited (Balfour *et al.*, 1983; Whitley *et al.*, 1992). The recommended dose of intravenous acyclovir for herpes zoster in severely immunocompromised patients is 10–15 mg/kg (or 500 mg/m^2) every 8 hours (Table 65.1). When the infection is under control, therapy can be switched from intravenous acyclovir to an oral antiviral drug for the remainder of the course of treatment. Patients should be treated until healing is complete or for a minimum of 10–14 days (whichever is longer) to reduce the risk of relapsing disease.

Treating shingles in immunocompromised patients on an outpatient basis with oral antiviral drugs is an attractive approach, although supporting data are limited. One small study randomized 27 allogenic HSCT recipients with herpes zoster to either oral or intravenous acyclovir. No VZV dissemination occurred in either group, and no differences in healing or clinical outcome were apparent (Ljungman *et al.*, 1989). Published data from clinical trials with famciclovir and valacyclovir for herpes zoster in immunocompromised patients remain limited, but a growing body of clinical experience suggests that these drugs are safe and effective in this setting (Tyring *et al.*, 2001a,b). For less severely immunosuppressed patients, oral therapy with acyclovir (800 mg five times daily), valacyclovir (1000 mg three times daily), or famciclovir (500 mg three times daily), coupled with close clinical observation, is a reasonable option. Because of the risk of ocular involvement, intravenous acyclovir plus evaluation by an ophthalmologist are recommended for highly immunocompromised patients who present with HZO.

HIV-seropositive patients

The incidence of herpes zoster is about 15-fold higher in HIV-seropositive men than in age-matched controls. Shingles in this population is associated with higher rates of CNS complications, necrotizing retinitis, and recurrent episodes. Prospectively acquired data to guide clinicians when selecting antiviral therapy for herpes zoster in HIV-seropositive patients are currently limited. Nearly 300 HIV-infected patients with shingles were enrolled in controlled studies comparing orally administered acyclovir with the investigational antiviral drug sorivudine. Overall, the time to cessation of new vesicle formation, total crusting, and resolution of zoster-associated pain were 3–4 days, 7–8 days, and about 60 days, respectively (Bodsworth *et al.*, 1997; Gnann *et al.*, 1998). These studies confirm the efficacy and safety of oral antiviral therapy for herpes zoster in patients with HIV infection. Valacyclovir and famciclovir have not been systematically evaluated as treatments for herpes zoster in HIV-infected patients, although anecdotal clinical experience suggests therapeutic benefit. Long term administration of antiherpes virus drugs to prevent recurrences of herpes zoster in patients with AIDS is not routinely recommended. Because of the documented risk of relapsing infection, VZV disease in HIV-seropositive patients should be treated until all lesions are completely resolved, which is often longer than the standard 7–10-day course. What impact anti-VZV therapy may have on the risk of subsequent complications such as CNS infection or retinitis is unknown. Adjunctive therapy of herpes zoster with corticosteroids has not been evaluated in HIV-infected patients and is not currently recommended.

On the basis of clinical experience, most physicians select intravenous acyclovir as the drug of choice to treat severe or complicated herpes zoster in HIV-infected patients. The literature contains numerous case reports documenting

successful therapy of neurologic complications with intravenous acyclovir (Poscher, 1994; Lionnet *et al.*, 1996). Some experts have recommended intravenous acyclovir for initial therapy of HZO in HIV-infected patients, although oral therapy appears adequate in most cases.

A syndrome of herpetic retinal necrosis can occur as a late complication of herpes zoster in either immunocompetent or immunocompromised patients, but is seen with the greatest frequency in patients with AIDS. Responses to intravenous acyclovir or ganciclovir have been inconsistent and disappointing. Several case reports have documented preservation of vision in patients treated with a combination of intravenous ganciclovir plus foscarnet, with or without intravitreal ganciclovir (Galindez *et al.*, 1996). The optimal duration of induction therapy and options for long-term maintenance therapy for acute retinal necrosis in HIV-seropositive patients have not been established (Ormerod *et al.*, 1998). When VZV retinitis occurs in immunocompetent patients, the clinical outcome is clearly improved by acyclovir therapy and the prognosis is better. In this population, a suggested treatment regimen based on clinical experience is intravenous acyclovir (10–15 mg/kg every 8 hours) for 10–14 days, followed by oral valacyclovir 1 gram po three times a day for 4–6 weeks (Palay *et al.*, 1991).

Clinical indications for prophylaxis

Varicella

Immunocompetent patients

Administration of varicella vaccine within the first few days after exposure to VZV will produce a protective (or partially protective) immune response in VZV seronegative individuals (Watson *et al.*, 2000). About half of patients receiving post-exposure immunization may still develop some signs and symptoms of chickenpox, but the disease manifestations are usually very mild. Postexposure vaccination appears to be more effective and less expensive than pre-emptive therapy with antiviral drugs. This approach may be useful for managing VZV exposures that occur in a family, in the workplace, or in a medical care setting.

Pregnant women

Advisory committees have recommended administration of varicella-zoster immune globulin (VZIG) to VZV-susceptible pregnant women who have been exposed to varicella (Centers for Disease Control and Prevention, 1996). For maximal efficacy, VZIG must be administered as soon as possible after exposure (within 96 hours). VZIG (as available in the United States) is administered by deep intramuscular injection at a dose of 125 units/10 kg of body weight, to a maximum of 625 units. Intravenous immunoglobulin also contains substantial titers of VZV-specific IgG and may be substituted if VZIG is not available. Unfortunately, in this time-critical scenario, the true VZV serologic status of a pregnant woman with a negative history of varicella is often not known. The clinician may be faced with a decision to initiate passive immunoprophylaxis empirically or to wait for the results of serologic testing. The ideal time to determine VZV serologic status is before pregnancy, when vaccination can be offered to women who are confirmed to be seronegative (Glantz and Mushlin, 1998). Varicella vaccination of pregnant women is not currently recommended because of the theoretical risk of the live virus vaccine for both the fetus and the mother. Prophylactic (or pre-emptive) therapy with acyclovir for a pregnant woman after VZV exposure may be effective, but is an unproven approach.

Immunocompromised (including HIV-seropositive) patients

VZV-seronegative immunocompromised patients with a defined close exposure to either chickenpox or herpes zoster should receive VZIG to provide passive immunity (Zaia *et al.*, 1983). In most cases, VZIG administration will not prevent infection in the susceptible host, but it will significantly reduce the severity of the resultant illness. Placebo-controlled trials in immunocompromised children have demonstrated that VZIG ameliorates the severity of chickenpox and that it significantly reduces the risk of disseminated infection. A single treatment reduces the risk of disseminated infection by about 75% and provides four weeks of passive immunity. VZIG must be administered within 96 hours of exposure at the dose described above. VZIG is not useful for the treatment of established varicella or herpes zoster. The efficacy of VZIG prophylaxis in HIV-seropositive children or adults has not been evaluated prospectively.

Prophylactic administration of acyclovir following VZV exposure has been studied to a limited extent in susceptible immunocompetent patients, but not in immunocompromised individuals. In studies of healthy children conducted in Japan, varicella developed in 16% of the children prophylactically treated with acyclovir and in 100% of children in the control group (Asano *et al.*, 1993) About 80% of children prophylactically treated with acyclovir subsequently seroconverted, indicating VZV infection without significant disease (Suga *et al.*, 1993). However, additional data are required before this approach of preemptive antiviral chemotherapy can be routinely recommended

in either immunocompetent or immunocompromised populations. A suggested (but unvalidated) regimen is acyclovir 200 mg orally four or five times daily for 21 days beginning five days after VZV exposure.

Concerns about the use of the live, attenuated VZV_{oka} vaccine in immunocompromised patients have focused on the potential for the vaccine virus to cause disease and on the possibility that immunocompromised patients will fail to mount a protective immune response. Limited experience with the vaccine in leukemic children and renal transplant recipients have demonstrated that it can be used safely in highly selected populations (Arbeter *et al.*, 1990; Furth and Fivush, 2002).

Herpes zoster

Immunocompetent patients

There are no circumstances that warrant antiviral chemotherapy to try to prevent herpes zoster in immunocompetent individuals. A live-virus vaccine has proven to be effective for preventing herpes zoster and reducing PHN (Oxman *et al.*, 2005). A randomized, double-blind, placebo-controlled clinical trial enrolling 38,546 adults (age 60 and over) was conducted to evaluate the live attenuated Oka/Merck VZV vaccine. The primary endpoint was "herpes zoster burden of illness," a composite score capturing zoster incidence, duration, and severity of total pain and discomfort. Compared with placebo, the vaccine reduced the zoster burden of illness by 61.1%, reduced the incidence of herpes zoster by 51.3%, and reduced the incidence of PHN by 66.5% ($P < 0.001$ for all comparisons). The vaccine was associated with mild reactogenicity (local erythema or tenderness) in 48.3% of recipients, but was otherwise well tolerated. The herpes zoster vaccine was approved for use in the United States in 2006 for immunocompetent adults 60 years of age and over.

Immunocompromised patients

Drug regimens designed to prevent HSV recurrences in immunocompromised patients undergoing cancer chemotherapy or organ transplantation will also effectively prevent herpes zoster (Ljungman, 2001). Combined results from two placebo-controlled trials of long-term (6 months) acyclovir prophylaxis in HSCT recipients demonstrated herpes zoster in 11 (18%) of 62 placebo recipients and in none of the 62 acyclovir treated patients (Lundgren *et al.*, 1985; Perren *et al.*, 1988). Interestingly, the incidence of zoster increased dramatically after the discontinuation of prophylaxis such that, 12 months after transplantation, the cumulative number of herpes zoster cases was virtually identical between the acyclovir and placebo groups. Nonetheless, acyclovir prophylaxis effectively prevents herpes zoster during the early post-transplant period when patients are most severely immunosuppressed and thus have the highest risk for VZV-related complications. Although transplant specialists almost universally recommend 3–6 months of acyclovir prophylaxis, no consensus currently exists regarding the relative merits of longer term prophylaxis. Development of a heat-inactivated VZV vaccine for use in immunocompromised patients is an area of active investigation (Hata *et al.*, 2002).

HIV-seropositive patients

Antiviral chemoprophylaxis for prevention of herpes zoster in patients with AIDS is not routinely recommended. A significant number of HIV-seropositive patients take suppressive antiviral drugs to prevent genital HSV reactivations, which may also prevent herpes zoster. In patients with multiple recurrent episodes of herpes zoster, chemoprophylaxis could be considered (e.g., valacyclovir 1 gram orally twice a day or famciclovir 500 mg orally twice a day), although this approach is unvalidated.

Drug-resistant varicella-zoster virus

Since first reported in 1988, multiple isolates of acyclovir-resistant VZV have been recovered from immunocompromised patients, usually HIV-infected individuals with very low CD4+ T-lymphocyte counts. The mechanism of resistance is based on the deletion or truncation of the gene expressing thymidine kinase. Most isolates resistant to acyclovir are also resistant to valacyclovir, famciclovir, penciclovir, and ganciclovir, all of which depend on viral TK for activation. A strong association exists between acyclovir-resistant VZV and the presence of atypical skin lesions (Boivin *et al.*, 1994; Levin *et al.*, 2003a,b). One report described four HIV-seropositive adults undergoing chronic suppressive acyclovir therapy who developed disseminated hyperkeratotic papules that failed to respond to acyclovir (Jacobson *et al.*, 1990). In vitro susceptibility testing confirmed that the VZV isolates were acyclovir-resistant with a mean IC50 for acyclovir of 20 μg/ml, compared with 0.75 μg/ml for the reference strain (VZV_{oka}). Although the mechanisms that lead to the development of acyclovir resistance are incompletely understood, clinical data indicate that many cases are associated with inadequate dosing of acyclovir for either acute therapy or long-term suppression, possibly allowing for selection of TK-deficient mutants. Clinicians using acyclovir or related drugs for treatment of varicella or herpes zoster in AIDS patients should

utilize the full therapeutic dose and continue therapy until all VZV lesions have completely resolved (Jacobson *et al.*, 1990).

The drug of choice for treatment of acyclovir-resistant VZV disease is foscarnet, an inhibitor of viral DNA polymerase that is not dependent on TK for activation (Breton *et al.*, 1998) (Table 65.1). In a series of 13 patients with AIDS and acyclovir-resistant VZV infections treated with intravenous foscarnet, 10 patients (77%) had complete lesion healing after a mean of 17.8 days of therapy (Breton *et al.*, 1998). Most cases of disease caused by acyclovir-resistant VZV have been limited to cutaneous involvement, although a few instances of visceral infection caused by acyclovir-resistant VZV have been reported, including cases of retinal necrosis and meningoradiculitis.

Fortunately, VZV isolates resistant to both acyclovir and foscarnet have been encountered infrequently. The molecular biology of these duly-resistant isolates has not been fully explored, but a mutation in the viral DNA polymerase can account for both acyclovir and foscarnet resistance. Cidofovir would likely retain activity against these isolates and would become the drug of choice for patients with disease caused by dually-resistant VZV.

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66

Antiviral therapy for human cytomegalovirus

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Introduction

The remit of this chapter is to summarize what is known about licensed antiviral drugs for CMV. In summary, we do not possess a single anti-CMV drug, which is potent and safe enough to be given to all individuals infected with this virus. What follows therefore, is the evidence-base for prescribing the existing compounds with the objective of maximizing therapeutic efficacy and cost-effectiveness while minimizing toxicity.

Licensed drugs and mechanism of action

Nucleosides

Ganciclovir (GCV) and acyclovir (ACV) are related nucleosides (see Fig. 66.1) which are anabolized by a common cellular pathway. After activation, they are competitive inhibitors of CMV encoded DNA polymerase. In cells infected with CMV, the first stage of phosphorylation is achieved by the UL97 protein kinase. Once GCV is monophosphorylated within the virus-infected cell, it is charged and so unable to diffuse out of the cell. A concentration gradient is thereby formed across the plasma membrane, aiding diffusion of more GCV into the infected cell. Cellular enzymes convert GCV monophosphate to the triphosphate. GCV triphosphate is a potent inhibitor of CMV DNA polymerase and has a long intracellular half-life. Selectivity for virus-infected cells is achieved both by UL97 activation and because GCV triphosphate is a better inhibitor of CMV-encoded DNA polymerase than cellular DNA polymerase.

Ganciclovir possesses a free hydroxyl at a position equivalent to the 3' of the open sugar ring and so can allow DNA elongation. This means that it is not an obligate chain terminator, although chain termination usually occurs after incorporation of one or more molecules. The ability to allow chain elongation is theoretically undesirable because it might occur in uninfected cells leading to a mutagenic event in cellular DNA; GCV in particular is oncogenic at low dosages in rodents due to this incorporation into cellular DNA.

ACV can also be activated by UL97 (Talarico *et al.*, 1999) and acyclovir triphosphate is a potent inhibitor of CMV DNA polymerase (Mar *et al.*, 1985) so that this compound can also inhibit CMV. Acyclovir triphosphate is an obligate chain terminator and also a suicide inhibitor of herpesvirus DNA polymerase (Furman *et al.*, 1984). In combination, these characteristics potently inhibit CMV replication. However, the intracellular half-life of ACV-triphosphate is significantly shorter than that of GCV-triphosphate so that high drug levels and frequent dosing are needed for ACV to control CMV replication in vivo (Lowance *et al.*, 1999). Selectivity for virus-infected cells is achieved by UL97 activation and because ACV triphosphate is a better inhibitor of CMV-encoded DNA polymerase than cellular DNA polymerase.

The oral absorption of GCV is poor while that of ACV is better but also variable between individuals. Oral bioavailability of these compounds has been improved via esters which are absorbed and then cleaved in the intestinal wall and/or liver to release free compound. Valganciclovir is the valine ester of GCV. Valaciclovir is the valine ester of ACV.

Nucleotides

Cidofovir (CDV) is a nucleotide. These compounds are phosphonates, structurally equivalent to the nucleoside monophosphate but without the charge which would prevent the molecule crossing the plasma membrane. Cidofovir bypasses the UL97 step and is converted to the diphosphate (equivalent to the nucleoside triphosphate; see

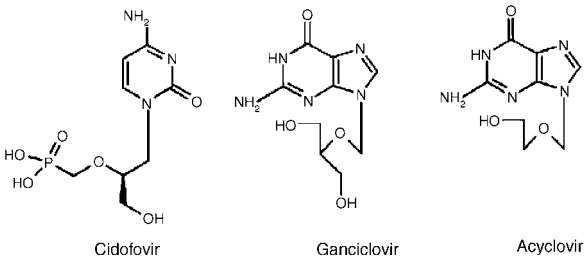


Fig. 66.1. Chemical structures of licensed antiviral drugs with activity against CMV in vivo.

Fig. 66.2) by cellular enzymes. The selectivity of CDV resides in the preferential inhibition of CMV DNA polymerase rather than cellular DNA polymerase by CDV diphosphate.

Foscarnet

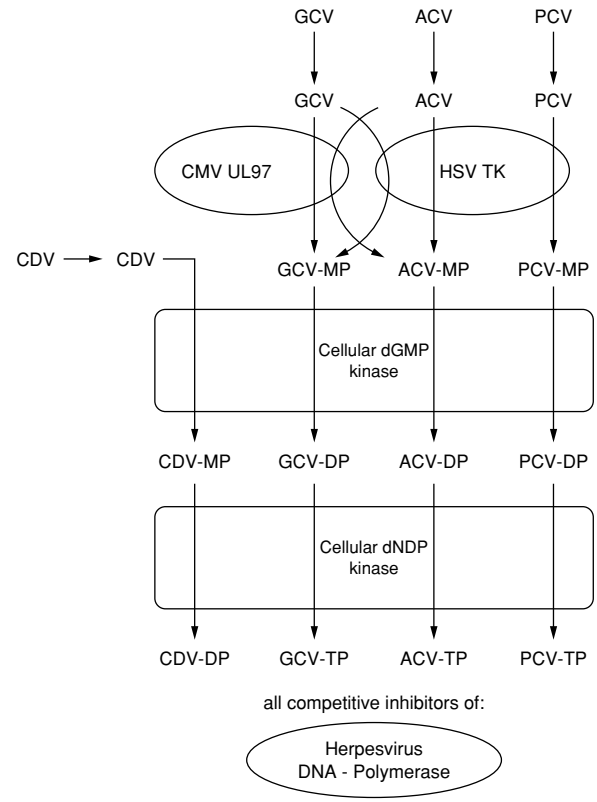
Pyrophosphate is eliminated when a phosphodiester bond is formed as a natural nucleoside triphosphate and is incorporated into a growing DNA chain. Foscarnet is structurally analogous to pyrophosphate and inhibits CMV DNA polymerase by binding to the enzymic site for pyrophosphate. Since pyrophosphate is one of the products of DNA enzyme activity, foscarnet is a product inhibitor, not a substrate inhibitor, and so does not compete with the natural nucleotides.

Fomivirsen

Molecules complementary (antisense) to mRNA bind mRNA to prevent expression of the encoded gene. For CMV, the antisense molecule fomivirsen binds to the major immediate-early transactivator gene. The half-life of this compound is long because it contains modified nucleosides such as phosphorothioates (substitution of sulfur into the phosphodiester background) and/or modified sugar residues, which are not readily degraded by host cell enzymes. In contrast to the other compounds mentioned above, fomvirsen is administered intravitreally only to the end-organ involved with CMV retinitis and so does not affect systemic CMV.

Clinical efficacy; challenges for achieving clinical benefit

Opportunistic infection with CMV occurs when a patient is immunocompromised because the immune system is immature (fetus/neonate), dysregulated (engrafting hematopoietic stem cell transplant), suppressed pharmacologically (solid organ transplant recipient) or suppressed by HIV infection (AIDS). The clinical benefits of treatment must therefore be seen in the context of these complex medical cases and require a detailed understanding of disease pathogenesis in each of the patient groups. Specifically, there is evidence (Rubin, 1991) that CMV has "direct effects" (where the virus can be seen histologically in the affected end-organ) and "indirect effects" where the virus is statistically associated with and may trigger conditions such as graft rejection, accelerated atherosclerosis, an immunosuppressive syndrome and death (not attributable to CMV end-organ disease). Antiviral treatment has the potential of inhibiting both direct and indirect effects but



Enzymes encoded by herpesviruses are shown as ovals.
Enzymes encoded by mammalian cells are shown as rectangles.

ACV = acyclovir
GCV = ganciclovir
PCV = penciclovir
MP = monophosphate
DP = diphosphate
TP = triphosphate
CDV = cidofovir
TK = thymidine kinase
UL97 = 97th gene in unique long region

Fig. 66.2. Anabolism of anti-viral drugs to make active forms.

Table 66.1. CMV diseases in the immunocompromised

Symptoms	Solid T_x	BMT/SCT_x	AIDS
Direct effects			
Fever/hepatitis	+++	+	+
Gastrointestinal	++	++	+
Retinitis	+	+	+++
Pneumonitis	+	+++	
Myelosuppression		+	
Encephalopathy		+	+
Polyradiculopathy			+
Addisonian			+
Indirect effects			
Immunosuppression	+	+	
Rejection/GvHD	+	?	
Atherosclerosis	+		
Death		+	+

carefully controlled clinical trials are required to prove this definitively.

All seropositive individuals should be assumed to possess latent CMV capable of reactivation. Thus, if they become immunocompromised, CMV frequently reactivates from latency and may cause disease (reactivation infection). In addition, organs harvested from a seropositive individual may transmit virus (Grundy *et al.*, 1988), irrespective of whether the recipient is seronegative (primary infection) or seropositive (reinfection).

Table 66.2. Implications of the CMV viral load results from 1975

CMV is a systemic infection
Sampling of urine can provide a paradigm of CMV replication in inaccessible target organs
There may be a threshold value of viral load above which disease becomes common
Antiviral therapy may have clinical benefits even if it cannot completely stop virus replication
A short duration of therapy may provide clinical benefit, even though virus excretion continues for years

Pathogenesis

Viremia and viral load

Most CMV end-organ disease in the immunocompromized is caused by viremic spread to multiple organs (Table 66.1). The risk of CMV disease correlates strongly with high CMV viral loads as first described in 1975 by Stagno and colleagues (Stagno *et al.*, 1975). They compared viruria in neonates with symptomatic congenital, asymptomatic congenital, or perinatal infection. The group with CMV disease had, on average, one log higher viruria than those with asymptomatic congenital infection, who in turn had an average one log higher viruria than those with perinatal infection (see Fig. 66.3). This observation has a series of implications for understanding pathogenesis and for focussing antiviral therapy (see Table 66.2). For example, the results suggested that there might be a threshold viral load above which CMV disease became common. This possibility has been investigated using

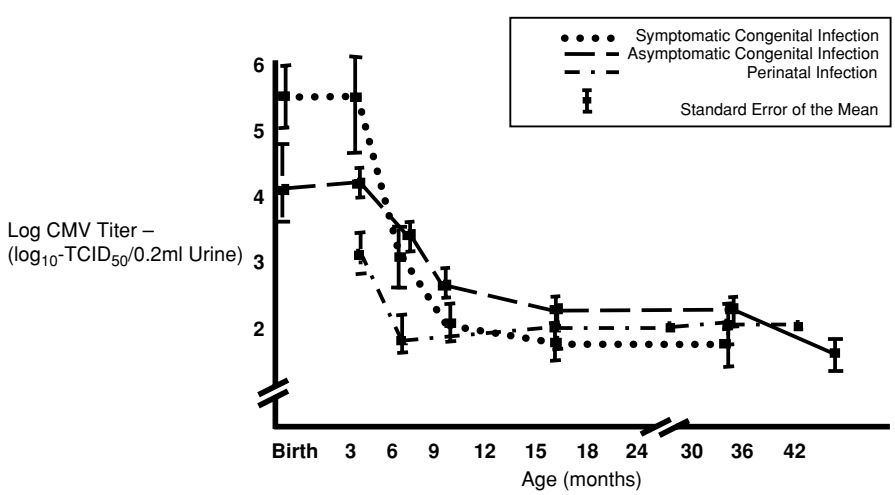


Fig. 66.3. CMV load From Stagno *et al.*, 1975.

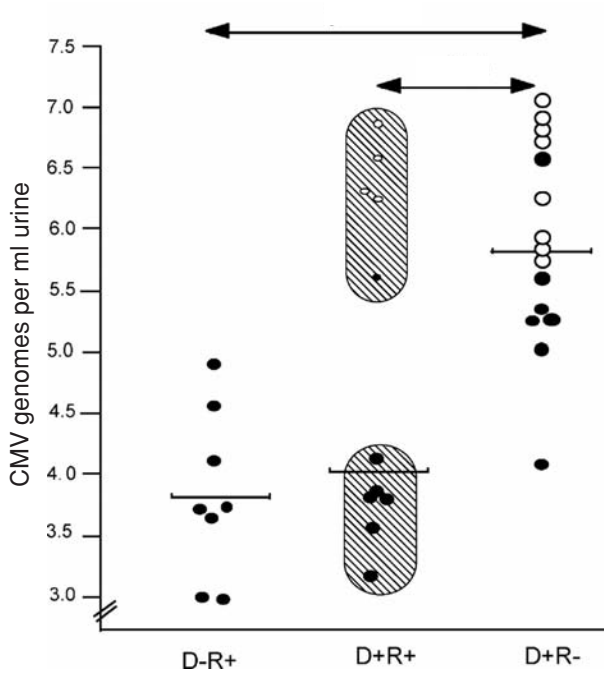


Fig. 66.4. Inter-relationships between peak CMV viral load, donor recipient serostatus and CMV disease in 35 patients with active CMV infection after renal transplantation. (Reprinted with permission from Cope, 1997b)

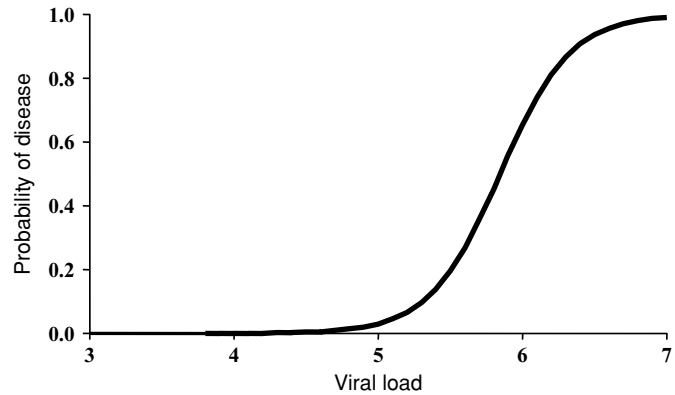


Fig. 66.5. The threshold concept From Cope, 1997b

quantitative-competitive PCR (QCPCR), which showed that, after renal transplant, a significant correlation is apparent (Cope *et al.*, 1997b) between the median values of maximum viruria post-transplant and the presence of CMV disease (see Fig. 66.4). The same is true when viral load is measured in blood (Hassan-Walker *et al.*, 1999) from renal transplant, liver transplant, and stem cell transplant patients, with CMV viral loads in the blood significantly greater in patients with CMV disease in each case (Cope *et al.*, 1997a,b; Gor *et al.*, 1998).

Donor/recipient serostatus at the time of transplant also identifies (Betts *et al.*, 1977) patients at risk of CMV disease (e.g. see Fig. 66.4). For recipients of solid organs, highest risk groups are D+R− (ie donor seropositive, recipient seronegative), followed by D+R+, then D−R+. These groups represent primary infection, reinfection plus reactivation and reactivation infections, respectively. For stem cell transplant recipients, the highest risk is D−R+, followed by D+R+, then D+R−. These groups correspond to reactivation, reactivation in the presence of marrow from immune donors and possible transmission of virus from donor marrow, respectively. In addition, multiple studies in all transplant patient groups identified viremia as a risk factor for CMV disease (Meyers *et al.*, 1990). Multivariate statistical analyses showed that for all patient populations, high viral load remained a risk factor for CMV disease after viremia and donor/recipient serostatus had been controlled statistically. In contrast, donor/recipient serostatus and viremia were no longer statistically significant once viral load had been controlled (Cope *et al.*, 1997a,b; Gor *et al.*, 1998) . Thus, high viral load is the major determinant of CMV disease and the classically defined risk factors of donor/recipient serostatus and viremia are markers of CMV disease because of their association with high viral load. In addition, the relationship between increasing viral load and disease is non-linear, with a threshold value above which CMV disease is much more common (Cope *et al.*, 1997a,b) (Fig. 66.5). This finding implies (Table 66.2) that potent prevention of disease could be achieved if drugs were deployed to prevent viral load exceeding critical values.

Serial measurements of viremia in several groups of immunocompromized patients demonstrated that CMV replicates with rapid dynamics, approximating to a doubling time (viral load increasing) or half-life (viral load decreasing) of one day (Emery *et al.*, 1999). The half-life is even shorter among patients experiencing primary infection (Emery *et al.*, 2002). This means that its reputation as a "slowly growing" virus is undeserved and that drugs of high potency are required to interfere with its replication. Thus, viral load measurements explain much of the pathogenesis of CMV disease and are important for understanding disease processes, for targeting the deployment of antiviral drugs, for measuring the success of antiviral therapy and for predicting the development of resistance (see later).

Disease processes within infected organs

Direct effects

Much of the end-organ disease caused by CMV can be attributed to *lysis*, i.e., destruction of cells as a direct result of virus replication. This can literally be seen clearly in the special case of the retinal cells destroyed by CMV (i.e. retinitis) but similar processes probably account for hearing loss, hepatitis, adrenalitis, gastrointestinal tract ulceration, encephalitis, and polyradiculopathy. In all of these cases, CMV can be seen histopathologically in biopsies, can be cultured from biopsies (showing productive replication) and responds to antiviral therapy. In contrast, some other diseases associated with CMV may be triggered by the virus, but may be caused by immune responses.

CMV pneumonitis appears typically in the second month post stem cell transplant after patients have had CMV viremia and asymptomatic CMV lung infection as shown by bronchial lavage at day 35 (Schmidt *et al.*, 1991). Thus, marrow engraftment, often associated with GvHD, represents a risk factor for CMV pneumonitis, implying that an aberrant immune response may contribute to disease (for review, see Grundy *et al.*, 1987). However, CMV can also occur before engraftment. Once established, CMV pneumonitis responds very poorly to ganciclovir alone, but the addition of immunoglobulin may give an improved response rate (Ljungman *et al.*, 1992). A cytokine-driven disease caused by abnormal cell-mediated effectors is suspected and, if correct, could explain why CMV pneumonitis is uncommon in AIDS patients with low CD4 counts. An alternative hypothesis is that the pulmonary toxicity of chemotherapy and/or irradiation causes damage which predisposes to viral pneumonia when viral replication occurs in the lung following transplantation (Schmidt *et al.*, 1991). A corollary to the former hypothesis (Grundy *et al.*, 1987) was that restoration of the immune deficit in AIDS patients with anti-retroviral medication, might trigger CMV pneumonitis. Although we are unaware of any such cases of CMV pneumonitis in AIDS patients given HAART, an inflammatory response to CMV is commonly seen in the *eye*, with corresponding increased levels of patient morbidity (Karavellas *et al.*, 1999).

Indirect effects

CMV is associated with an increased incidence of acute graft rejection. The presumed pathogenesis involves CMV infection of the transplanted organ acting like a transplantation antigen, marking the organ for immune attack. Evidence for CMV playing this pathogenic role includes statistical association (Grattan *et al.*, 1989), detection of CMV in organs undergoing rejection, apparent response of late rejection to ganciclovir therapy in an uncontrolled study (Reinke *et al.*, 1994), and a significant reduction in biopsy-proven acute graft rejection among patients randomized to high-dose valaciclovir in a placebo-controlled trial of prophylaxis after renal transplant (Lowance *et al.*, 1999).

CMV is also associated with accelerated atherosclerosis after heart transplantation (Grattan *et al.*, 1989). CMV persists in monocytes/macrophages that could be attracted to sites of graft atheroma, either bringing CMV to that site or facilitating the formation of foam cells laden with oxidized lipids (Guetta *et al.*, 1997). CMV major immediate-early protein binds p53 in arterial smooth muscle cells (Speir *et al.*, 1994) suggesting that CMV could reduce apoptosis leading to proliferation of such cells. The US28 gene of CMV encodes a chemokine receptor which, once transferred experimentally to smooth muscle cells, confers the ability to migrate towards a source of chemokines. Thus, CMV infection might stimulate chemotactic mobility of these cells towards a site of inflammation (Streblow *et al.*, 1999). Finally, CMV stimulates the formation of reactive oxidized intermediates and could contribute further to the progression of atherosclerosis (Speir *et al.*, 1996). Irrespective of the mechanism(s), follow-up of heart allograft patients who took part in a placebo-controlled trial of GCV reported reduced accelerated atherosclerosis among those allocated the drug (Valantine *et al.*, 1999), so replicating in humans what had previously been shown with rat CMV in rats (Lemstrom *et al.*, 1997).

CMV infection is associated with an increased incidence of bacterial or fungal superinfection (Falagas *et al.*, 1996; Nichols *et al.*, 2002) follow-up of the heart allograft patients mentioned above demonstrated reduced fungal infection in those randomized to receive GCV (Wagner *et al.*, 1995). This implies that CMV is functionally immunosuppressive and possible mechanisms have been reviewed recently (Boeckh and Nichols, 2003). If CMV does contribute to the net level of immuosuppression, this could explain why it is associated with EBV-induced lymphoproliferative disease. It could also explain why AIDS patients with first episode CMV retinitis have a significantly higher mortality rate if their CMV viral load in blood is above the median of the whole group of patients (Bowen *et al.*, 1996) and why the death rate (in the pre-HAART era) was associated more strongly with CMV viral load than with HIV viral load (Spector *et al.*, 1999). Recent results show that this same effect is still present in the era of HAART (Deayton *et al.*, 2004).

Clinical manifestations of CMV end-organ disease

The major clinical manifestations of CMV disease in different groups of immunocompromised patients are summarized in Table 66.1. These should be defined with

reference to the criteria laid down at the International CMV Workshop (Ljungman *et al.*, 2002) which include: compatible clinical features plus signs of end-organ dysfunction plus demonstration of CMV in the affected organ (exception retina). In particular, diseases should be described in terms of the body system affected and the term "CMV syndrome" should be avoided.

Fever/leukopenia

CMV viremia is often associated with prolonged spiking fever (e.g. $>38\,^{\circ}C$ on two consecutive days), with or without leukopenia. These constitutional symptoms may resolve spontaneously or may lead to end-organ disease. Other causes of fever (e.g., bacteremia) and leukopenia (e.g., doses of immunosuppressive drugs) must be excluded. CMV-related marrow graft failure has been described as a rare complication after stem cell transplant.

Hepatitis

Transaminases may be raised (e.g., $>2.5\times$ upper limit of normal), with or without alkaline phosphatase representing an obstructive component. Hyperbilirubinemia may be present but frank jaundice is uncommon. Hepatitis usually resolves spontaneously but may herald other end-organ disease. It is predominantly seen in solid organ transplant recipients, with a predeliction to liver transplantation.

Gastrointestinal disease

CMV may involve the gastrointestinal tract anywhere from the mouth to the anus. The presentation is usually with pain, often accompanied by fever. Esophagitis, odynophagia and abdominal pain mimicking perforation indicate involvement of the esophagus/colon, respectively. Endoscopy reveals mucosal ulcerations, with or without *Candida* superinfection. The ulcers respond slowly to anti-CMV treatment and may perforate and/or hemorrhage.

Retinitis

This can occur in any immunocompromised patient but is most common in AIDS. Symptoms, if present, include "floaters," flashing lights, and/or loss of central vision. Small peripheral lesions may be unnoticed by the patient; lesions involving the macula may be imminently sight-threatening and demand immediate treatment. Involvement of a large proportion of the retina interrupts retinal/scleral attachment and represents a risk factor for retinal detachment. Before the availability of HAART, most patients had progression of retinitis and the goal of treatment was to preserve vision (for review, see Jacobson, 1997). Treatment with HAART may be followed by vitritis (see Pathogenesis section), with paradoxical impaired vision despite better control of retinitis. Retinitis is also seen in the neonates born with symptomatic congenital CMV infection and rarely in transplant recipients (Crippa *et al.*, 2001).

Encephalitis

In AIDS patients, CMV reaches the brain by one of two routes; extension of a neighboring endotheliitis or via the choroid plexus. In the former case, the encephalitis follows a subacute course, difficult to distinguish from HIV dementia. In the latter, necrotizing ventricular encephalitis produces cranial nerve defects, nystagmus and ventriculomegaly (reviewed in Griffiths and McLaughlin, 1997). In both cases, response to treatment is poor. Encephalitis is a rare complication in stem cell transplant recipients.

Polyradiculopathy

An AIDS patient with a very low CD4 count presents with subacute weakness of the lower limbs, with or without bladder paralysis. Lumbar puncture reveals abundant polymorphonuclear leukocytes in the CSF. Immediate treatment is indicated but the clinical response is poor.

Pneumonitis

Most cases occur after stem cell transplantation with or without concurrent graft versus host disease (see Pathogenesis section). There is rapid onset of dyspnea plus hypoxia. Chest X-ray may be relatively clear initially but progresses to show interstitial infiltrates. Co-infection with aspergillus is common in stem cell transplant recipients. There is a high mortality, with poor response to treatment (Ljungman *et al.*, 1992).

Hearing loss

This may be apparent at birth in neonates born with symptomatic congenital CMV infection. It is also clear that hearing may be normal at birth but may deteriorate months or years later, irrespective of whether symptoms were present at birth. The hearing loss may be bilateral or unilateral.

Laboratory diagnosis

Detection of viremia

This can be performed by any published method which has been shown to provide a good positive predictive value for CMV disease, e.g. 50%–60% for the patient population to

Table 66.3. Strategies for chemotherapy of CMV

Term used	When drug given	Risk of disease	Acceptable toxicity	Treatment decision prompted by:
True prophylaxis	Before active infection	Low	None	Clinician
Delayed prophylaxis	Before active infection but after rejection	Medium	Low	Clinician
Suppression	After peripheral detection of virus	Medium	Low	Laboratory
Pre-emptive therapy	After systemic detection of virus	High	Medium	Laboratory
Treatment	Once disease apparent	Established	High	Both

be followed. Thus, the rapid diagnostic techniques using cell culture amplification testing of virus (termed DEAFF testing in Europe (Griffiths *et al.*, 1984) and shell-vial in the USA (Gleaves *et al.*, 1984) are no longer sufficiently sensitive and should be replaced with newer methods. Examples include PCR in whole blood (Kidd *et al.*, 1993), PCR in plasma (Spector *et al.*, 1992), ultrasensitive quantitative plasma PCR assays (Boeckh *et al.*, 1996) and antigenemia (The *et al.*, 1990). Recent results demonstrate that PCR from whole blood is superior to either PCR in plasma or peripheral blood leukocytes (Razonable *et al.*, 2002) and has the advantage of not requiring separation of blood specimens with the attendant risks of contamination and mis-labeling. A randomized trial has shown PCR to be superior to conventional cell culture for deciding when to initiate pre-emptive therapy (Einsele *et al.*, 1995). Laboratory protocols differ and it is important that all aspects of each method are followed in detail including sample processing and virus detection. These have been optimized to avoid the detection of latent virus while providing good sensitivity for predicting disease **not** necessarily the highest sensitivity for detecting asymptomatic infection (see Fig. 66.5). Thus, it is not possible to "mix and match" different aspects of PCR protocols.

CNS involvement

PCR of CSF is the method of choice for diagnosing CMV CNS infection (Shinkai *et al.*, 1995).

DEAFF/Shell vial

This method is still sufficiently sensitive and robust to diagnose CMV lung infection using bronchoalveolar lavage fluid. Cells from this fluid can also be cytocentrifuged and stained with monoclonal antibodies but this approach, while more rapid, lacks sensitivity compared to DEAFF/shell vial amplification. The assay is also very well suited to detect CMV in biopsy specimens (e.g., lung, gastrointestinal tissue).

Histopathology

This is performed on tissue biopsies to detect classic Cowdry type A intranuclear "Owl's eye" inclusion bodies. It is insensitive and so has a high specificity for disease (Mattes *et al.*, 2000).

Cell culture

This is performed on tissue biopsies after tissue is minced and inoculated directly on to cells. It is slow but sensitive.

Serology

Many enzyme immunoassays are commercially available for the detection of CMV IgG antibodies pre-transplant in both donor and recipient. Serologic testing has no role to play post-transplant.

Clinical indications for antiviral prophylaxis and dosage regimens

Management

The principles of managing CMV infection and disease in the immunocompromised host are to anticipate their development, to define policies for monitoring patients routinely for the presence of viremia according to their baseline risk of CMV disease, and to enhance surveillance if patients develop a condition likely to increase their risk of CMV disease. Using the principles of evidence-based medicine, the patient will then be offered prophylaxis or pre-emptive therapy based upon an assessment of their individualized risk of disease, together with data from controlled clinical trials in the same patient group supporting the efficacy and safety of possible antiviral interventions.

Strategies for deploying antiviral agents

Different strategies have been devised for controlling CMV disease based on the efficacies and toxicities of the drugs available at present (summarized in Table 66.3).

True prophylaxis

This strategy should be followed where baseline risk of disease is high, the chance of severe disease is also high and where at least one double-blind, randomized, placebo-controlled trial supports the efficacy and safety of prophylaxis in the target population. The drug is given from the time of transplant onwards (or from the time of engraftment in the case of GCV in stem cell transplants) and continued for the duration studied in the controlled clinical trial which provided evidence for its use. This is termed "true prophylaxis" because, from a virologic perspective, it administers the drug before there is active virus replication. By giving the drug early, prophylaxis may provide clinical benefits even if the drug has relatively low antiviral potency.

Delayed prophylaxis

At baseline, a decision was made that true prophylaxis was not indicated. However, the patient's situation has changed eg because augmented immunosuppression is required to control an episode of graft rejection and so it is decided to start prophylaxis now (Hibberd *et al.*, 1995). This is still termed "prophylaxis" because the drug is given before there is active virus replication and a drug with relatively low potency may be used.

Suppression

Some laboratories monitor weekly samples of urine and/or saliva from transplant patients and process them by a method shown to provide a moderate positive predictive value for CMV disease, e.g., 30% (Kidd *et al.*, 1993). If CMV is detected, an antiviral drug is given with the intention of suppressing virus replication below the level needed to cause viremia. A drug with moderate potency is required to keep CMV suppressed.

Pre-emptive therapy

This term describes intervention when the results of laboratory tests indicate that a patient is at imminent risk of CMV disease (Rubin, 1991). Nowadays it refers to detection of viremia in any immunocompromised patient but in the past was also used when asymptomatic lung infection was detected after stem cell transplantation (Schmidt *et al.*, 1991). In the first example, the patient should be monitored by collecting weekly samples of blood processed by laboratory methods known to provide a high positive predictive value for CMV disease, e.g., 50%–60% (Kidd *et al.*, 1993). The objective is to give an antiviral drug with the intention of halting CMV viremia before it reaches the high viral loads required to cause disease. A drug with high potency is therefore required.

Decision-points for starting pre-emptive therapy must be based upon the results of clinicopathologic studies with the assay under evaluation. Examples include qualitative detection of viremia by PCR or antigenemia where the assay level of detection has been shown to be associated with a high risk of disease, or two consecutive samples PCR-positive. Alternatively, if samples are processed by real-time quantitative PCR, then any value above a pre-determined cut-off should trigger antiviral intervention. The results of viral dynamic assessments can also be applied to this problem; patients at risk of disease can be identified by the absolute value of viral load found in the first PCR-positive sample, coupled with an assessment of individual viral dynamics by calculating the rate of increase from the last PCR-negative sample (Emery *et al.*, 2000). All of these approaches work well in clinical practice and comparative studies are required to determine if any one of them is superior to the others.

The treatment of neonates born with symptomatic congenital CMV infection also fits into this category because the objective is to prevent new end-organ damage.

Treatment of established disease

When a patient meets the case definition of CMV disease because he/she has compatible symptoms and signs, together with detection of CMV in the affected organ, a highly potent drug is required which will penetrate the affected organ and resolve the disease, including any immunopathologic components.

Results of double-blind, randomized, placebo-controlled trials

Results of published trials of licensed drugs defined according to these criteria are given in Table 66.4. It will be seen that the most potent drug *in vitro*, ganciclovir, has been subjected to several such clinical trials while foscarnet and cidofovir have not. Other agents such as interferon-alpha, acyclovir, valaciclovir and immunoglobulin, have also been evaluated although they were traditionally not thought to have useful anti-CMV activity.

For the endpoint of CMV infection, Table 66.4 shows that, in addition to ganciclovir (Balfour *et al.*, 1989; Cheeseman *et al.*, 1979; Lui *et al.*, 1992), interferon alpha (Cheeseman *et al.*, 1979; Lui *et al.*, 1992; Ljungman *et al.*, 1992) and acyclovir (Lowance *et al.*, 1999; Balfour *et al.*, 1989; Prentice *et al.*, 1994) have activity against CMV in vivo. The only two studies not to show an effect were the two studies of

Table 66.4. Double-blind, placebo-controlled, randomized trials of CMV

Significantly reduced CMV infection/disease/indirect effects					
Strategy	Drug	Bone marrow	Renal	Heart	Liver
Treatment	GCV	Reed (1990)			
Pre-emptive	GCV				*Paya*
Suppressive	GCV	**Goodrich** (1991)			
Prophylaxis	Interferon		Cheeseman (1979) *Hirsch* (1983) Lui (1992)		
	ACV	***Prentice*** (1993)	*Balfour* (1989)		
	VACV		**Lowance** (1999)		
	Ig		(Metselaar) (1989)		SNYDMAN (1993)
	GCV	Winston (1993) *Goodrich* (1993)		**Merigan** (1992) *Macdonald* (1995)	*Gane* (1997)

Font used for name of first author indicates significant benefit for the following endpoints:
Plain = reduced infection.
Italics = reduced infection plus reduced disease.
Plain Bold = reduced infection plus reduced disease plus reduced indirect effects.
Italics Bold = reduced infection plus reduced indirect effects.
Capitals = reduced disease.
Name in brackets – no effect.

immunoglobulin (Metselaar *et al.*, 1989; Snydman *et al.*, 1993). This implies that, if immunoglobulin has a role in the prophylaxis of CMV disease, it may not work by inhibiting CMV replication.

For the endpoint of CMV disease, Table 66.4 shows that ganciclovir failed to demonstrate a significantly better resolution of established CMV disease than placebo (Reed *et al.*, 1990). Part of this disappointing outcome may be attributed to the low dose (2.5 mg/kg t.i.d.) and/or short duration used (14 days) to treat gastrointestinal disease in stem cell transplant patients (Reed *et al.*, 1990). Nevertheless, it illustrates the difficulty of treating established CMV disease and so argues that the other strategies, which aim to prevent CMV disease, should always be pursued in preference to waiting for disease to present. Ganciclovir did reduce CMV disease when used in the suppressive mode for stem cell transplant patients (Goodrich *et al.*, 1991). It also had a significant benefit when used in one (Goodrich *et al.*, 1993) of two trials of prophylaxis after stem cell transplant; the second study (Winston *et al.*, 1993) showed a strong trend in favor of GCV which just failed to reach conventional statistical significance. Ganciclovir also significantly reduced CMV disease following prophylaxis given orally to liver transplant patients (Gane *et al.*, 1997) and intravenously to heart transplant patients (Macdonald *et al.*, 1995; Merigan *et al.*, 1992). However, benefit after heart transplant was evident only in the low risk group, with no effect in the D+R− group of one study (Merigan *et al.*, 1992), whereas the opposite outcome was seen in a second (Macdonald *et al.*, 1995). This difference might result from the longer treatment course in the latter study, together with a design difference such that patients experiencing rejection were given additional doses of GCV. Prophylactic ACV significantly reduced CMV disease after renal transplant (Balfour *et al.*, 1989), as did prophylactic valaciclovir (Lowance *et al.*, 1999). In a prophylaxis trial after stem cell transplantation, acyclovir significantly decreased CMV viremia and showed a non-significant trend towards reduced CMV disease (Prentice *et al.*, 1994). A trial of immunoglobulin prophylaxis showed reduced "CMV syndrome" in liver transplant recipients despite having no significant effect on CMV infection (Snydman *et al.*, 1993). Sub-group analysis showed an effect on CMV-associated fungal superinfection (part of the pre-defined "CMV syndrome") so it remains possible that the immunoglobulin predominantly reduces fungal rather than CMV infection. CMV-specific immune globulin and a gH monoclonal antibody were ineffective in stem cell tranplant recipients (Boeckh *et al.*, 2001; Bowden *et al.*, 1991).

Table 66.4 also summarizes the impact of these drugs on the indirect effects of CMV infection. The number of deaths in the solid organ transplant populations is too low to provide the statistical power to address this endpoint. After stem cell transplant, GCV significantly improved survival when used suppressively (Goodrich *et al.*, 1991). However, when used prophylactically, no effect was seen (Goodrich *et al.*, 1993; Winston *et al.*, 1993). This was not a problem of small sample size and neither study demonstrated even a trend in favor of ganciclovir. The most likely explanations are that (i) some patients with viremia still received preemptive therapy (Goodrich *et al.*, 1993) so reducing CMV-induced mortality in both arms (ii) neutropenia induced by prophylactic GCV predisposed patients to succumb to bacterial or fungal superinfections, so mitigating the potential benefits of this drug. Overall, these studies indicate that GCV is too toxic a compound to be used for prophylaxis after stem cell transplant, although it is literally life-saving

when used in a suppressive mode (Goodrich *et al.*, 1991). This illustrates that, in prophylaxis, all patients are exposed to side-effects and that suppression or preemptive therapy, by limiting the number of patients exposed to the drug, can produce an enhanced therapeutic ratio. In contrast, ACV prophylaxis after stem cell transplant produced a survival benefit (Prentice *et al.*, 1994), presumably because its more modest efficacy was not offset by serious toxicity. After renal transplant, VACV produced a marked reduction in biopsy-proven acute graft rejection corresponding to a 50% decrease in incidence among seronegative recipients at risk of primary infection (Lowance *et al.*, 1999). The effects in seropositive recipients were smaller, implying that CMV (rather than another herpesvirus susceptible to the drug) is responsible for this indirect effect and that most CMV-induced graft rejection occurs in the subset of patients with primary infection. Following heart transplantation, GCV significantly reduced fungal infections (Wagner *et al.*, 1995) and accelerated atherosclerosis (Valantine *et al.*, 1999).

Table 66.5 summarizes randomized studies which compared two anti-CMV strategies. Boeckh *et al.* (1996) compared antigenemia-guided preemptive therapy with ganciclovir prophylaxis at engraftment and found a higher CMV disease rate at day 100 with pre-emptive therapy; however, there was no statistically significant difference at day 400 between the two groups. Invasive bacterial and fungal infections were more common with GCV prophylaxis, resulting in similar mortality rates at day 400 (Boeckh *et al.*, 1996). Humar *et al.* compared GCV based on a surveillance BAL at day 35 with antigenemia-guided preemptive therapy and found these two strategies equivalent in a small randomized trial (Humar *et al.*, 2001). Reusser *et al.* compared foscarnet with GCV for preemptive therapy in stem cell transplant recipients (Reusser *et al.*, 2002). Survival without CMV disease was similar between the groups, however, GCV caused more neutropenia. Foscarnet was associated with more electrolyte imbalances but renal insufficiency was no different between the two groups. One trial by Winston *et al.* compared valacyclovir with ganciclovir prophylaxis in stem cell transplant recipients. The incidence of CMV disease was similar in both groups, however, the trial was not large enough to make meaningful conclusions (Winston *et al.*, 2003). Another small trial by Winston *et al.* compared sequential intravenous and oral ganciclovir with intravenous ganciclovir for 3 months in D+/R− liver transplant recipients (Winston and Busuttil, 2004). The incidence of CMV disease was not statistically different between the groups.

In liver transplant patients, prophylaxis with GCV is superior to prophylaxis with ACV (Winston *et al.*, 1995).

Table 66.5. List of randomized trials of two strategies (for results see text; includes open-label trials)

Strategy	Drug	Bone marrow	Renal	Heart	Liver
Prophylaxis	ACV				Winston
vs.					*et al.*, 1995
Prophylaxis	GCV				
Pre-emptive	GCV	Boeckh *et al.*,			
vs.		1996			
Prophylaxis	GCV				
Suppression	GCV	Humar *et al.*,			
vs.		2001			
Pre-emptive	GCV				
Preemptive	FSC	Reusser *et al.*,			
vs.		2002			
Pre-emptive	GCV				
Prophylaxis	GCV	Winston *et al.*,			
vs.		2003			
Prophylaxis	V-ACV				
Prophylaxis	GCV (oral)				Winston and
vs.					Busuttil
Prophylaxis	GCV (iv)				2004
Prophylaxis	GCV (oral)		Paya *et al.*,	Paya *et al.*,	Paya *et al.*,
vs.			2004	2004	2004
Prophylaxis	V-GCV				

A large randomized double-blind trial of valganciclovir versus oral GCV in D+/R− solid organ transplant recipients showed similar rates of CMV disease with the two compounds (Paya *et al.*, 2004). Neutropenia was observed more frequently with valganciclovir. In another randomized trial in AIDS patients with CMV retinitis valganciclovir showed similar activity to intravenous ganciclovir for treatment of retinitis (Martin *et al.*, 2002).

Additional randomized trials compared different methods of detecting CMV in blood for preemptive therapy and found equivalence between antigenemia and pp67 mRNA (Gerna *et al.*, 2003a,b) and IE mRNA (Gerna *et al.*, 2003a,b). An earlier randomized trial by Einsele *et al.* established that PCR-based detection by CMV viremia is superior to culture-based detection of viremia for initiation of preemptive therapy (Einsele *et al.*, 1995). A comparison of ganciclovir plus foscarnet (each at half dose) compared to full dose ganciclovir showed that these two drugs are not synergistic in humans when used for pre-emptive therapy (Mattes, 2004).

Aspects of CMV management in HIV-infected individuals

The introduction of highly active antiretroviral therapy (HAART) for HIV has dramatically changed the

Table 66.6. Relative merits of prophylaxis vs. pre-emptive therapy

Proposed advantages of prophylaxis
Proven benefit controlled clinical trials
• *CMV disease*
• *indirect effects*
Avoids complicated logistical problems
• *real-time laboratory assays*
• *organization of sample collection*
• *geographical location*
Protects against HSV, VZV
Overall, may be more cost-effective
• *costs of laboratory tests*
• *indirect effects CMV*
• *other herpesviruses*
Proposed advantages of pre-emptive therapy
Target resources on patients most at need (financial, skill)
Treat when viral load lower
• *shorter treatment*
• *reduced recurrences*
• *reduced resistance?*
Allows low level stimulation of immunity
• *reduces late-onset disease?*
Protects patients non-compliant with prophylaxis
It is possible that PET may also reduce indirect effects

From Snydman, 2006, Singh, 2006.

significance of CMV in HIV-infected individuals. There is now good evidence that in patients with CMV retinitis who have a sustained response to HAART, CMV viremia disappears promptly (Deayton *et al.*, 1999) and maintenance treatment can be discontinued without relapse of retinitis. Several groups have reported HAART-naïve patients with good CD4 (>150/μl) and HIV virologic responses (Lin *et al.*, 2002) to therapy who have been able to stop anti-CMV maintenance medications completely without disease progression (Whitcup *et al.*, 1999). Thus, intravenous or oral therapy (i.e., Valganciclovir (Martin *et al.*, 2002) can be used until the anticipated return of the patient's CMV-specific immunity. CMV disease can still occur within 4 months of HAART initiation despite adequate suppression of HIV replication (Jacobson *et al.*, 1997). Some patients also develop immune recovery vitritis when HAART is initiated at the time of CMV retinitis (Karavellas *et al.*, 1999; Whitcup *et al.*, 1999). Alternatives to intravenous and oral antivirals for the treatment of CMV retinitis are available. In the current era, intraocular implants are used primarily in salvage regimens (disease progression with first-line agents) or in patients for whom immunologic recovery with HAART is not anticipated.

Conclusions

1. Decisions about which drugs to recommend for particular treatment strategies must draw upon evidence-based medicine provided by the results of controlled clinical trials. Decisions must consider toxicity as well as efficacy.
2. For stem cell transplant patients, prophylaxis with ACV (Prentice *et al.*, 1994) or VACV (Lowance *et al.*, 1999) is recommended; however, virologic monitoring and pre-emptive therapy are still required to allow GCV to be administered (Goodrich *et al.*, 1991). Since the latter study showed that, of the samples of urine, saliva, and blood processed, only blood provided prognostic value, this conclusion probably applies to pre-emptive therapy as well.
3. The toxicity problems of GCV apply specifically to the stem cell transplant population where GCV and VACV have been shown to be equally potent in practice (Winston *et al.*, 2003). A head-to-head comparison of low dose GCV versus ACV for prophylaxis after liver transplant shows that GCV is superior (Winston *et al.*, 1995).
4. Prophylaxis and pre-emptive therapy are both effective strategies for preventing CMV disease. Their relative merits are hotly debated (see Table 66.6).
5. Similar data from AIDS patients cannot be presented because, remarkably, no trials have been designed based on these virologic criteria. Two trials (Feinberg *et al.*, 1998; Spector *et al.*, 1996) have been conducted of clinical prophylaxis but these are not the same as true prophylaxis because, at the time of randomization, although no patients had CMV disease, some of them would be expected to have asymptomatic infection. Indeed, the subsequent virologic studies show that oral GCV had its greatest effect when given to the subset receiving true prophylaxis and had little effect in the subset receiving pre-emptive therapy (Spector *et al.*, 1998). In contrast, the virologic studies of VACV demonstrated that this drug had its greatest effect when given for pre-emptive therapy (Griffiths *et al.*, 1998). This might be thought to imply that VACV is more potent in vivo than oral GCV but a randomized head-to-head comparison would be needed to test this possibility. Such a trial is unlikely to be conducted because the high dose of VACV chosen (2 g q.i.d.) was poorly tolerated by the AIDS patients (Feinberg *et al.*, 1998), although the same dose was safe when studied in renal transplant patients

(Lowance *et al.*, 1999). Furthermore, the incidence of CMV disease in the current era of HIV management has declined due to immune reconstitution following initiation of HAART.

6. The ACV and VACV studies do show that potent inhibition of CMV DNA polymerase by ACV-TP triphosphate can have clinical utility under some circumstances. In the renal transplant study, the authors provide evidence that plasma levels of ACV were higher than expected because of poor renal clearance, but were still lower than would be required to inhibit CMV based on in vitro data (Fletcher *et al.*, 1991), which demonstrates clearly that the IC50 levels produced by fibroblast cell cultures are misleadingly high.
7. Although the incidence of CMV end-organ disease has been dramatically reduced in both transplant and HIV patients, the indirect effects of CMV remain important. Recent data in unrelated donor or T-cell depleted stem cell transplant recipients demonstrate that preemptive therapy alone, although quite effective in reducing the risk of CMV disease, has not eliminated the survival disadvantage associated with CMV seropositivity (Boeckh and Nichols, 2004). In AIDS patients, CMV viremia remains an important factor for poor outcome in the era of highly-active antiretroviral therapy (Deayton *et al.*, 2004). Indeed, once CMV viremia was accounted for in multivariable models, HIV viral load was no longer associated significantly with death (Deayton *et al.*, 2004).
8. In the case of neonates born with symptomatic CMV infection with CNS involvement, a study by Kimberlin *et al.* (2003) shows that the proportion who develop progressive hearing loss can be decreased by a six week course of GCV at 6 mg/kg i.v. bid. Although this study was not placebo-controlled (due to the ethical problems of administering an intravenous placebo to neonates), the results of this seminal study are included here because they are clinically important and entirely consistent with the pathogenetic implications of neonatal viral load (see Fig. 66.3 and Table 66.2).

Resistance

Resistance to GCV maps to two genetic loci, UL97 and DNA polymerase. In general, changes in UL97 are common and confer low level resistance while changes in DNA polymerase are rare but some of them confer cross-resistance to other antiviral drugs.

Methods used to define resistance

It is important to note that both UL97 and DNA polymerase are large genes in which spontaneous polymorphisms exist. It is not therefore possible to sequence the gene from a patient, find a genetic change compared to the standard laboratory strain, AD169, and then conclude that resistance has developed. A mutation identified in a patient sample must be examined by marker transfer (site-directed mutagenesis) before it can be concluded that the mutation is responsible for conferring resistance. In the process of marker transfer, the genetic sequence is altered in a laboratory-adapted strain and it is proven that this change confers resistance to the antiviral drug by analyzing the phenotype in plaque reduction tests. To demonstrate that no additional changes have been introduced by mistake, the resistant strain should then be back-mutated to the wild type and it should be proven that sensitivity to the antiviral drug has returned. Given the slow evolution of cytopathic effect in vitro, these experiments are time-consuming, inefficient and expensive. In addition, the requirement for selection by means of ganciclovir is undesireable because it offers the potential of introducing additional changes at the in vitro passage level. However, recent technical changes have made ganciclovir selection now no longer necessary so it should become easier in the future for marker transfer experiments to be performed. These technical advantages include a particular strain that has been developed with a novel restriction enzyme site, the ability to use cosmid clones of the genome to reconstitute infectious viruses and the recent cloning of CMV as a bacterial artificial chromosome (Adler *et al.*, 2003; Chou *et al.*, 2002). All of the mutations described in the sections which follow have been identified in clinical samples and proven by marker transfer experiments to confer resistance.

Gene UL 97

Gene UL97 produces a serine/threonine kinase enzyme which is essential for virus replication. Through studies with a gene deleted virus, and with an experimental drug (maribavir) which inhibits gene UL97, it was shown that UL97 is important for nuclear egress (Krosky *et al.*, 2003). It has also recently been shown that UL97 phosphorylates elongation factor 1 delta at codon serine 133. This is exactly the biochemical change made by the cellular CDC2 plus cyclin B when they prepare the nuclear envelope for dissolution just prior to mitosis. It is therefore tempting to suggest that UL97 mimics this cellular pathway to prepare the cell to release into the cytoplasm virions synthesized in the nucleus (Kawaguchi and Kato, 2003).

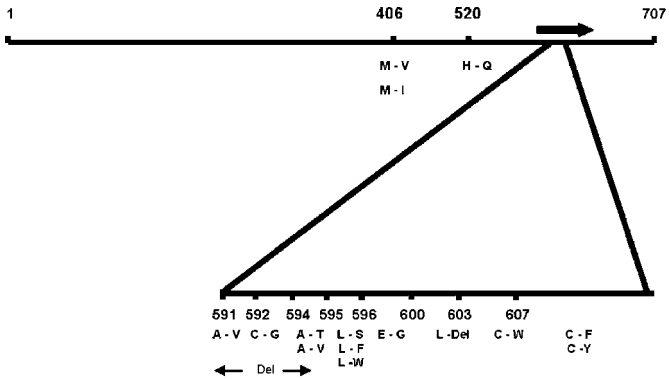


Fig. 66.6. UL97 mutations conferring GCV resistance. (From Chen, 2002.)

Figure 66.6 shows the mutations which confer ganciclovir resistance in gene UL97. Many of these individual mutations have been transferred to recombinant vaccinia viruses and shown to significantly reduce phosphorylation of ganciclovir compared to wild-type. However, the reductions in phosphorylation are rather low (e.g., 10% reduction for the deletion of 590–593). If CMV replication continues in the presence of ganciclovir then additional mutations can occur in UL97 which do not themselves confer resistance but act to partially restore the efficiency of the enzyme. For example, by transferring the individual mutations into recombinant vaccinia viruses, Chou and colleagues were able to show that D605E compensates for an A594P mutation (Chou *et al.*, 2000).

When these UL97 mutants are propagated in vitro they appear to have identical replication kinetics in one step growth curves (Chou *et al.*, 2002). This makes it very difficult to identify the true significance of these mutations in vivo and represents one of many deficiencies of studying this virus in fibroblasts (for review, see Griffiths, 2002). Fortunately, the high rate of replication demonstrated in patients explains how CMV variants resistant to GCV can evolve, and provides a basis for calculating their relative fitness compared to wild type (Emery *et al.*, 1999). These mathematical modelling techniques can also be used to explain and predict the circumstances under which resistant strains become prominent (Emery and Griffiths, 2000). In summary, short courses of GCV are unlikely to select resistant strains, but long or repeated courses, especially with oral GCV, provide ideal opportunities for resistant strains to flourish. They also demonstrate why resistant strains are cultured infrequently in practice; the process of incubating mixed populations of strains for 3–4 weeks in cell cultures lacking GCV allows the wild-type virus to out-compete the mutant strain, leading to the incorrect conclusion that resistance is not present in vivo.

Gene UL54 (DNA polymerase)

Fig. 66.7 shows a schematic representation of the DNA polymerase gene showing the important catalytic sites and areas where resistance to ganciclovir or foscarnet or cidofovir have been mapped. In contrast to the finding with UL97 mutants, changes in UL54 often confer a growth disadvantage upon virus replication in vitro (Chou *et al.*, 2003) which may help explain the infrequency of their detection in patients.

Although the three-dimensional structure of CMV DNA polymerase is not known, important insights into its mechanism of action can be gleaned by comparison with the known structures of related enzymes (Chou *et al.*, 2003). Changes at the exonuclase site (Fig. 66.7) probably facilitate pyrophosphorolysis (i.e., the reverse reaction of DNA polymerization) which results in the excision of an incorporated drug moiety. Changes in region V interfere with the normal function of "thumb" (GCV/CDV cross-resistance) and "fingers" pyrophosphate exchange and acceptance of the incoming nucleotide (GCV/Foscarnet cross-resistance). Changes at other regions of the enzyme may reflect subtle conformational effects on these or other functions of the enzyme. At least one such change can confer hypersensitivity to foscarnet, a phenomenon which was first described for HSV but which is now frequently encountered in HIV.

Patterns of cross-resistance

Given the structural similarity of, GCV and ACV (Fig. 66.1), it would be expected that virus resistant to one compound exhibits cross-resistance to the other. This is difficult to prove because fibroblast cell cultures do not demonstrate the sensitivity of CMV to ACV, let alone its resistance. In practice, GCV has been used so widely that the small number of resistant strains available for study have been selected through the use of this drug and all of these viruses should be assumed to exhibit cross-resistance to ACV.

If strains of CMV resistant to GCV due to mutations in UL97 are encountered in clinical practice and alternative treatment is required then foscarnet is the first choice. Cidofovir is an alternative which, by bypassing UL97, has the advantage of avoiding the genetic change commonly selected for under GCV pressure (Safrin *et al.*, 1997).

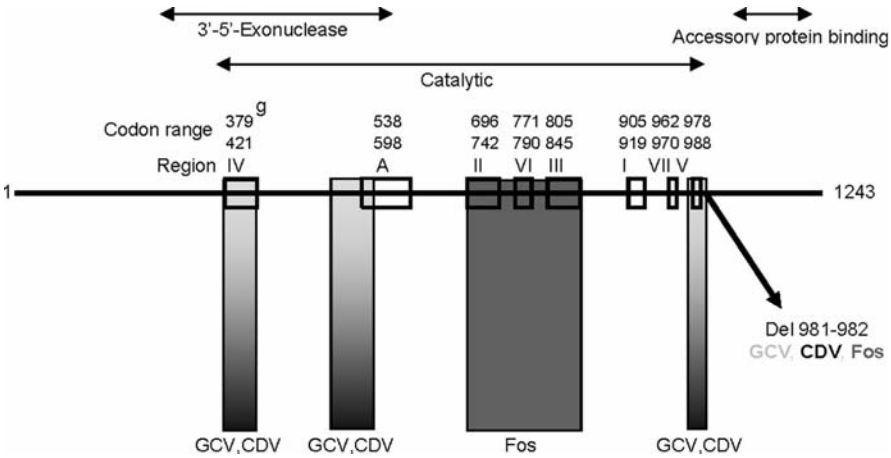


Fig. 66.7. Mutations in CMV DNA polymerase which confer antiviral resistance. (From Chou, 2002, 2003.)

If resistance to GCV has been acquired through mutations in UL54 then precise details of the genetic changes are required. Many of the changes confer cross-resistance to cidofovir (see Fig. 66.7) so this drug is not usually indicated. Foscarnet remains the drug of choice for UL54 mutants; although one particular mutant exhibits low-level cross-resistance to ganciclovir, cidofovir and foscarnet.

Recognition of resistance in clinical practice

The early studies of CMV in AIDS patients receiving chronic GCV therapy were important because they provided laboratory strains to allow a definition of biochemical resistance in vitro (Drew *et al.*, 1991; Erice *et al.*, 1989). However, they underestimated the incidence of resistance because viruses from samples of urine or blood were propagated in cell cultures in vitro. Most such clinical samples contain mixed populations of wild-type and resistant strains of CMV. As explained earlier, the UL97 mutants have decreased fitness in the absence of GCV (Emery *et al.*, 1999). As a result, they are readily out-competed by wild strains in the laboratory, especially given the requirement for serial passage in vitro in order to provide high titer inocula for the plaque reduction test. This problem can be bypassed by either collecting samples from patients with CMV end-organ disease, which are less likely to contain mixed populations of virus, or by using molecular techniques to detect genetic markers of resistance directly in the patient samples without passage in vitro.

Bowen *et al.* used a point mutation assay and sequencing to identify GCV resistance in 10/45 (22%) AIDS patients with CMV retinitis (Bowen *et al.*, 1998). Limaye and colleagues (Limaye *et al.*, 2000) examined blood isolates from 25 cases of CMV disease in recipients of solid organ transplants and found GCV resistance in 5 (20%). Liu *et al.* (2000) showed that virtually all CMV DNA extracts from the eyes of untreated AIDS patients had wild type UL97 sequences, consistent with the concept that UL97 mutants have impaired fitness in the absence of GCV. Hu *et al.* (2002) identified 13 (15%) UL97 mutations in vitreus samples from 87 AIDS patients and found the same mutations in the blood of 11 of these patients, showing that changes in

Table 66.7. Management algorithm for suspected resistant CMV

When to suspect drug resistance

1. Drug-naïve patient:
   - If viremia fails to disappear with preemptive therapy
     - culture-positive > 3 weeks
     - increasing antigenemia levels > 2 weeks
     - increasing PCR levels > 2 week
   - If viremia appears during prophylaxis
     - Any culture positivity
     - Increasing level for > 2 weeks

Diagnostic

Obtain blood PCR or DNA from site and sequence UL97 (UL54)

Drug therapy:
- *Check compliance/dose/schedule*
- *swap to alternative antiviral agent*

General
- Reduction of immunosuppression if feasible (in severe cases or resistant CMV disease "sacrificing" the transplanted organ may be considered or necessary)

If toxicity appears:
- *GCV/foscarnet each at half-dose*
- Novel compounds needed

blood strains frequently mirror those in the eye. Boivan et al used restriction fragment length polymorphisms and direct sequencing of UL97 to detect resistant strains in approximately 12% of AIDS patients receiving valganciclovir and showed that UL97 sequencing directly from the blood correlated well with disease progression (Boivin *et al.*, 2001). They also showed UL97 sequence discordance when the same samples were passed in cell culture, consistent with the concept that cell cultures preferentially select wild type isolates during passage in vitro (Gilbert and Boivin, 2003).

It should now be clear that resistance to GCV has been underestimated in the past. New drugs targeted at different CMV genes are required (see Chapter 68) and randomized controlled trials are essential to define their efficacy.

The basic principles to help avoid the development of resistance are to reduce CMV replication in the presence of drug (i.e., treat with potent compounds and adequate doses for a short time and then stop therapy) and to treat promptly at an early stage (when the virus load is low) (Table 66.7). Serially monitored patients with increasing viral loads over several weeks are at high risk of having drug resistance, especially after prolonged prior exposure to the drug. Ideally, treated patients should be followed with serial quantitative measures of CMV viral load to demonstrate when CMV replication has been controlled and so optimize when treatment can be stopped (or an alternative antiviral drug used). Randomized controlled clinical trials are required to determine whether such individualization of therapy duration confers advantages over prescription of a course of standard length.

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New approaches to antiviral drug discovery (genomics/proteomics)

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Introduction

Discovery of antiviral drugs has always been an opportunistic endeavor. Small molecules in general and nucleoside analogues in particular have led investigators to discover uncharacterized viral gene products that could be exploited for the purpose of antiviral chemotherapy. Great strides have also been made in understanding fundamental events in the viral replication cycle including the binding of viral glycoproteins to cellular receptors, viral regulatory proteins that control expression of viral and cellular gene expression, viral genes that affect the synthesis and packaging of the viral genome, and viral factors that subvert the host immune response (Whitley and Roizman, 2001). Many of the viral genes that contribute to these processes are known and for some of them, the precise function is understood at the molecular level. For these targets it is comparatively simple to reduce the essential function to a biochemical assay, such as a polymerase or protease assay for use in a high throughput screen in order to identify small molecule inhibitors of enzyme function (Liu and Roizman, 1993). This approach has facilitated the proactive and rational search for specific enzyme inhibitors and has led to the development of effective antiviral therapies. Although this approach is effective, it requires well-characterized targets with a defined biochemical function, and can be applied only to a very small proportion of the essential viral gene products. At present, the best targets for antiviral chemotherapy likely remain undescribed and unutilized.

The development of new classes of antiviral drugs is limited by our understanding of biology. More often than not, it is unclear which viral gene products contribute to essential functions. Less clear still is the precise function or functions that the constituent proteins perform such that the biochemical activity can not be modeled in vitro. Thus, the challenge and rate-limiting step in this process is to identify the functions of gene products that are required for viral replication and to define precisely the molecular mechanisms involved with these processes.

Advances in genomics and associated technologies are offering new opportunities to investigators to answer such questions. This endeavor is driving technological developments in bioinformatics, genetics and laboratory automation and promises to generate a host of new resources that can be brought to bear on all fields of biology (Collins *et al.*, 2003). Advances in molecular virology will help drive this effort and will also benefit tremendously as virus–host interactions are defined in more detail and on a grander scale. Genomics, proteomics and related technologies can also be applied specifically to the discovery and development of antiviral therapies. Techniques and approaches that are particularly suited to this task are discussed and early experiments in this arena will be described.

Development of bioinformatics and computational tools

The emerging and evolving fields of genomics and proteomics are changing the way that biological research is conducted and technologies associated with these efforts can be applied effectively to the study of viral biology. The development of these fields is driven in part by the technological advances that produce genomic sequences more efficiently every year (Venter *et al.*, 2003). Significant technological advances in chemistry, biotechnology, and bioinformatics are also major drivers in this field and each of these efforts has produced tremendous new resources and even greater quantities of raw data. Perhaps the most

defining characteristic of these emerging fields of research is the tremendous size and complexity of the data sets they generate. Significant computational resources are required to manage the volumes of data, manipulate it, and parse it into databases that can be queried by researchers. This process requires training in computer science and mathematics. Investigators who specialize in these fields will contribute greatly to any future drug discovery projects using these new technologies. Bioinformatics resources are also required to analyze the data, identify patterns and display the patterns in a way that can be interpreted by investigators in the field. Data that is processed in this manner is designed to help investigators understand the problem at hand and can help them make hypotheses. Inferences drawn from these efforts need to be tested and confirmed in the laboratory, but the process helps to focus valuable research time on prioritized compounds or genes.

The application of computational methods to the study of herpesviruses biology is particularly intriguing. Large DNA viruses present a unique set of well characterized genomes that are comparatively well studied and characterized (Davison *et al.*, 2002). New tools developed by scientists in bioinformatics can be applied to these genomes to test their ability to predict the organization of viral genes, their global coding capacity and the function of viral proteins (Novotny *et al.*, 2001; Rigoutsos *et al.*, 2003). Laboratory confirmation of transcriptional patterns and gene expression and gene function is essential to this process, particularly early on when the bioinformatics algorithms are being tested on these genomes. The genetic tractability of herpesviruses and availability of genome wide methods to test these hypotheses make this an ideal system to validate new approaches to study biological processes (Stingley *et al.*, 2000). Communication and cooperation between the specialists in bioinformatics and bench level virologists is essential and will facilitate the incremental improvement of algorithms predictive of biological structure and function. The iterative process of algorithm refinement and biological confirmation will lead to computational approaches that are increasingly predictive of viral biology.

Studying new methods in bioinformatics in herpesviruses presents certain virus specific problems. Even within the alphaherpesviruses, the variation in G + C content will present a codon bias that must be taken into consideration. The genomic organization of 3′ coterminal transcription units as well as readthrough of certain stop codons will complicate the computational analysis as well as laboratory characterization of gene expression. Indeed, this is true in other viruses, such as SV40 where algorithms trained on mammalian genomes fail in many cased because of conservative assumptions about splicing sites and polyadenylation signals. Nevertheless, the genomics studies at the level of herpesviruses will be particularly instructive and will guide efforts to define and interpret sequences from more complex genomes, such as the human genome.

Data relevant to the discovery of new drugs tend to be concentrated at the intersection of large data sets. Databases that contain information related to biological function, chemical structure, and the biologic activity of small molecules all can contribute to the search for new lead compounds. The nature of this problem is inherently complex and databases will be required to handle the volumes of data. In the near term, computational methods can suggest aspects of viral replication that might be targeted specifically by small molecule inhibitors. Similarly, these methods may identify small molecules with well described biological effects that could be used to probe cellular functions that may be required by the virus. In the long term, computational approaches have the potential to link databases containing information on chemical structure, protein structure, biochemical activity, and biologic activity of small molecules. It may eventually be possible to mine existing data in a meaningful way to suggest chemical classes that might be used to inhibit known biochemical activities.

Impact of genomics and related fields on herpesvirus research

The scientific landscape has changed dramatically in the last 15 years with the completion of the DNA sequence for the genomes of all eight known human herpesviruses (Baer *et al.*, 1984; Chee *et al.*, 1990; Davison and Scott, 1986; Dolan *et al.*, 1998; Gompels *et al.*, 1995; McGeoch *et al.*, 1988, 1991; Pfeiffer *et al.*, 1995; Russo *et al.*, 1996). This is a very significant step in the path towards understanding the biology of herpesvirus infections. These and other resources in the publicly available databases are tremendously valuable to scientists studying herpesvirus infections and provide a map and common reference points to help scientists describe precisely viral transcripts and open reading frames (ORFs). Genomic sequences can also be used to compare genomic organization among all the herpesviruses, and represents a starting point in the path towards the identifying evolutionary relationships in this virus family. Importantly, the nucleotide sequences in the databases are not static. Sequence data are inherently noisy and most genomic sequences in GenBank have mistakes that need to be corrected when they are identified, especially in information-dense viral genomes. Annotations of

viral genomes are conducted with the best tools available at the time of submission, but they become outdated as gene prediction algorithms improve and as experiments in the laboratory identify new genes (Cha *et al.*, 1996). Since the annotation process is evolving, scientists should expect to find inconsistencies among the annotations of different genomes, as the terminology used to describe gene function is continually evolving (Ashburner *et al.*, 2000). Thus, prototype viral genomes will need to be updated and reannotated in an iterative process, particularly as new strains and viruses are sequenced (Davison *et al.*, 2003). Versioning of genomes and annotations is becoming more important when comparing sequences and annotations due to the continual annotation process, analogous to the versioning of software and human genome release versions in NCBI.

Genomics studies in herpesviruses initially focused on defining the structure and coding capacity of each of the viral genomes and comparing them with annotated sequences of related viruses. The DNA sequence and structure of the genomes is comparatively simple to define, yet even for these simple organisms it is not possible to predict with any certainty which, if any genes will be expressed in the context of an infection. Depending on the algorithms used, different numbers of ORFs will identified and laboratory experimentation is required to confirm which ORFs are actually expressed in the context of a viral infection (Davison *et al.*, 2003; Rigoutsos *et al.*, 2003). As synthesized microarrays become less expensive, oligonucleotide probes which hybridize to putative exons and splice junctions can be used for confirming the expression of predicted transcripts and splice variants in a bulk fashion (Shoemaker *et al.*, 2001). Continued experimentation and consistent reannotation among the submitted sequences will help define how these viruses, and indeed their human host use DNA to regulate and code for all the required functions. As this knowledge base expands, relationships among all the herpesviruses will crystallize and molecular evolutionary patterns will start to emerge.

The viral proteome represents all of the proteins expressed by the virus and reflects both the processing of RNA transcripts as well as the post-translational processing that occurs. Proteomics methods have the potential to be particularly powerful because it can distinguish the modification of viral gene products during the course of viral infection and can help characterize how proteolysis, glycosylation and phosphorylation impact viral replication. At one level, mass spectrometry and protein microsequencing can be used to identify sets of viral proteins involved in a particular biologic process (Greco *et al.*, 2001). Genome wide searches using algorithms to predict protein structure or identify conserved motifs can also be used to generate hypotheses regarding protein function (Oien *et al.*, 2002). Yeast two-hybrid studies provide an experimental approach for the study protein-protein interactions among viral and cellular proteins and will help to provide information about functional complexes in an infected cell. Efforts underway to construct protein-protein interaction maps have the potential to help understand which gene products cooperate in biological processes.

The emerging field of chemical genetics will also likely impact the discovery of antiviral therapies and could be one of the most useful tools (Strausberg and Schreiber, 2003). Genes can be classified in orthologous groups (Tatusov *et al.*, 1997) and small molecules can be classified into families based on the chemical structure. Genetics can be used to characterize the phenotype associated with a particular gene, and in an analogous manner the biological effect of a drug, or chemotype, can be associated with small molecules. Relating chemotypes and genotypes can help identify the molecular targets and pathways affected by groups of compounds. This information can be used to infer the mechanism of action of candidate molecules by comparing the chemotype with existing phenotypes associated with viruses containing mutations in different pathways. This process might also be particularly useful in identifying cellular response patterns associated with drug toxicity that could be used to eliminate lead compounds early in the discovery process (Waring *et al.*, 2002).

New resources for use in drug discovery

Genomics and proteomics are promising new fields with lofty goals, but can they provide immediate utility to efforts currently underway to identify new classes of antiviral drugs? At present, the impact of these fields is most apparent in the widespread use of data and tools provided by the publicly available databases. The GenBank, EMBL, and DDBJ nucleotide databases provided freely on the web are a tremendous resource to everyone and most researchers use the databases or related tools on a regular basis. The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the European Bioinformatics Institute (http://www.ebi.ac.uk/services/index.html) websites in particular provide access, to basic tools and services that are used in laboratories every day. These tools include nucleotide and protein database searching tools, genome maps, structural databases and pattern recognition tools. The STDGEN database provided by Los Alamos National Laboratories (http://www.stdgen.lanl.gov/) is a wonderful resource that provides specific genomic and proteomic information on

herpesviruses and contains BLAST search results with links to other viral orthologues and conserved orthologous groups. Tools provided by these organizations are continually upgraded and new tools appear on a regular basis. Thus, a review of the available tools will be quickly outdated and can not substitute for a trip to the web sites listed in this text.

A number of new material resources have also been created through the automation of laboratory procedures. Microarrays of several different types are commercially available for characterizing changing transcription patterns in infected cells (Browne *et al.*, 2001). Herpesvirus specific microarrays have also been constructed and can rapidly assess viral transcript changes in response to stimuli (Stingley *et al.*, 2000). Expression clone libraries have been produced in efforts designed to assay gene function and virus knockout libraries have been assembled by a number of investigators. Each of the resources described here has the potential to be used in a genome wide search to identify viral genes that are important in the replication process.

Application of new technologies to cell-based antiviral assays

New tools and resources described herein, have the potential to be extremely useful in the drug discovery process and some strategies are currently in use. Many of the new strategies are unproven, and as such, are high risk activities that are used sparingly in the industrial setting. Nevertheless, high risk–high reward strategies have their place in the discovery process, particularly in the search for new classes of antivirals. The specific application of new technologies and resources to conventional screening and development activities has the potential to make an immediate and positive impact on the drug discovery process.

Cell-based screens for small molecules with antiviral activity have been utilized for all of the herpesviruses. Historically, most approved therapies for herpesvirus infections resulted from this approach to antiviral discovery, but continued screening of the same libraries with very similar assays is not likely to identify new lead compounds. It is possible to change the assays to bias the hit compounds away from molecules already identified in previous screens and towards molecules with different mechanisms of action. For example, screening recombinant viruses that lack the thymidine kinase (in herpes simplex virus (HSV)), or recombinant viruses lacking the *UL97* kinase (in cytomegalovirus (CMV)) will identify small molecules and nucleosides that do not require phosphorylation to be active. Lead compounds identified in these particular screens would also be candidates for the treatment of drug resistant infections. Genetically sensitized viruses, like the recombinants described above, might react more strongly to weakly active compounds and could unmask these molecules in existing chemical libraries. Candidate molecules with unusual mechanisms of action or good pharmacologic properties could then be selected for chemical modification to improve the antiviral activity.

As an alternative to the conventional endpoint of cytopathic effect inhibition, the affect of small molecules on viral transcriptional patterns could be monitored to reveal molecules with unusual mechanisms of action. Microarray technologies would be particularly useful in this regard, but it limits the number of compounds that could be examined in detail. In essence, this approach has already been validated through the characterization of gamma or late viral transcripts by treatment with phosphonoacetic acid (Stingley *et al.*, 2000). The cellular transcriptional response to small molecules could also be monitored simultaneously to measure changes in the host response to infection induced by the small molecules (Browne *et al.*, 2001; Fruh *et al.*, 2001). Microarrays can also help identify the mechanisms of transformation in infected cells and could potentially lead to better therapies for virus induced malignancies (Moses *et al.*, 2002). Potential toxicities could also be identified at an early stage by monitoring the induction of cellular genes associated with the response to toxic compounds (Waring *et al.*, 2002). Data generated by such an approach could also be queried at a later date using chemical genetics techniques to classify and cluster the chemotypes of molecules in the library.

Application of new technologies to biochemical assays

Biochemical screening assays will likely derive the greatest benefit from genomics and proteomics technologies. The increased characterization of viral gene products will likely identify additional biochemical activities that can be converted rapidly into small molecule screens. Homology searching algorithms also play a major role because of their ability to extend knowledge from one herpesvirus to related viruses. BLAST searches with a known gene can identify orthologues in other herpesviruses and related genes (paralogues) within the same virus in an effort to identify viral proteins with a similar biochemical activity (Davison *et al.*, 2002). For example, an orthologue of the HSV protease was identified in CMV and a similar biochemical assay could be used for both to screen for inhibitors of this biochemical activity (Jarvest *et al.*, 1999; Pinto *et al.*, 1999).

Many viral gene products have no known function, yet possess characteristic motifs that could potentially be used in biochemical screens (Rigoutsos *et al.*, 2003). Hypotheses generated by searches such as these could be tested in the laboratory and if confirmed could be used in a biochemical screen. For instance, nucleotide binding motifs in poorly characterized genes could be used to identify nucleosides that bind to these sites with high affinity. Similarly, a structural search revealed that *UL57* in CMV appears to be related at a structural level to some endonucleases (Novotny *et al.*, 2001). If this activity were confirmed in the laboratory, a biochemical screen could be used to identify specific inhibitors of this activity and might be effective in inhibiting viral replication given that this gene is known to be essential for viral replication.

Biochemical assays based on protein-protein interactions are capable of identifying molecules with antiviral activity. Information garnered from proteomics methods or yeast-two hybrids could be used to devise a rapid assay for protein–protein interactions that are presumed to be important for viral replication. Screening of small molecule libraries or peptide libraries could identify specific inhibitors that also possess antiviral activity (Liuzzi *et al.*, 1994). This same strategy could be employed to identify inhibitors that disrupt the interaction of viral and cellular proteins that appear to be important viral replication. If fact, it may be possible to perform the high throughput screen directly in yeast if the interaction was originally defined in this system.

The best characterized molecular targets have had their crystal structures determined. Given these data, it is possible to recrystallize these molecules with known inhibitors to identify protein binding sites or to dock small molecules *in silico* to identify molecules in improved binding affinities to the active site (Stoll *et al.*, 2003). This approach is particularly useful in lead optimization and medicinal chemistry efforts to increase potency. For any of the technologies discussed here, their greatest utility is generating testable hypotheses that could identify new and unexploited targets for antiviral therapy.

Application of new technologies to functional assays

Genetic approaches can identify gene products that are essential for viral replication, but without a biochemical assay, it is not possible to screen for inhibitors of these molecules. Functional genomics approaches can be employed to try to identify surrogate phenotypes for interesting genes. With these sorts of assays, the surrogate assays do not necessarily measure the relevant functional aspect of the gene in question. Any inhibitors identified from screens based on surrogate assays need to be validated in secondary assays (Tugendreich *et al.*, 2001). Although surrogate approaches are high risk high reward propositions, they are particularly useful for screening ion channels or other molecules that require an intact membrane for activity (Hahnberger *et al.*, 1996).

Application of new technologies to characterize mechanism of action and spectrum of activity

New technological developments also impact how researchers conduct preclinical studies on compounds with antiviral activity. Mechanism of action studies still involve the isolation of drug resistant viruses, but genomic sequencing of drug resistant viruses can be faster than conducting conventional marker transfer studies to identify the molecular targets of investigational drugs (S.W. Chou, pers. commun.). Direct sequencing is also cost effective in identifying resistance mutations in clinical isolates where the mechanisms of drug resistance are well characterized (Lurain *et al.*, 2001).

Genomics approaches can also be used to help predict the spectrum of activity of a compound if the mechanism of action is known. Homology searches can be used to identify gene families common to a wide variety of organisms and are called clusters of orthologous groups (COGs) (Tatusov *et al.*, 1997). This process was also conducted for all sequenced herpesviruses and a set of viral COGs was assembled (Montague and Hutchison, 2000). In addition to helping define orthologues in this group of viruses, the COGs can also be used to construct genome-wide phylogenetic trees, that closely match trees constructed using the highly conserved core genes (Davison *et al.*, 2002). COGs can also be clustered based on the viruses in which they appear, and can help to define a theoretical spectrum of activity for antiviral drugs based on the conservation of the molecular targets. A comparison of the predicted drug efficacy (shaded area) is compared with antiviral activity reported in the literature (+ or −) in Table 67.1. A number of interesting disparities are immediately apparent. Penciclovir did not appear to be active against EBV, and neither acyclovir nor penciclovir exhibited significant antiviral activity against HHV8, despite the fact that they possess relatively well conserved thymidine kinases. These results are explained in part by apparent low activity of the HHV8 thymidine kinase and narrow substrate specificity (Gustafson *et al.*, 2000). The activity of ganciclovir in viruses without TK orthologues can be explained

Table 67.1. Predicted spectrum of activity based on molecular target conservation

Drug	Molecular target	COG #(43) (Identity)	HSV1	HSV2	VZV	EBV	HHV8	CMV	HHV6A	HHV6B
Acyclovir	Thymidine kinase[a] (15, 23)	COG97 (18.9%)	+ (23)	+ (23)	+ (4)	+ (12)	− (31)	− (29)	− (37)	− (37)
Penciclovir	Thymidine kinase[a] (22)	COG97 (18.9%)	+ (6)	+ (6)	+ (6)	− (37)	− (37)	− (6)	− (37)	− (37)
Ganciclovir	Thymidine kinase[a] (15, 23)	COG97[a] (18.9%)	+ (39)	+ (39)	+ (14)	+ (39)	+ (31, 42)	+ (39)	+ (37)	+ (37)
	Protein kinase[a] (34, 55)	COG60[a] (17.2%)								
Foscarnet	DNA polymerase (10)	COG51 (27.6%)	+ (62)	+ (32)	+ (2)	+ (16)	+ (37)	+ (37, 62)	+ (37)	+ (37)
Cidofovir	DNA polymerase (20)	COG51 (27.6%)	+ (20)	+ (20)	+ (20)	+ (20)	+ (37)	+ (20)	+ (37)	+ (37)
4-oxo-dihydro-quinolines	DNA polymerase (46)	COG51 (27.1)	+ (57)	+ (57)	+ (57)	+ (57)	+ (57)	+ (57)	− (57)	− (57)
BDCRB	Terminase (UL89) (60)	COG59 (22.8%)	− (58)	− (58)	− (65)	− (58)	− (65)	+ (58)	− (65)	− (65)
	Terminase (UL56) (33)	COG93 (20.5%)								
maribavir	UL97 (5)	COG60 (17.2%)	− (65, 66)	− (65, 66)	− (65)	+ (66)	− (65)	+ (66)	− (65)	− (65)

[a] The molecular target is the polymerase (COG51), but the compounds derive most of their selectivity at the level of phosphorylation by the COGs shown.

by the alternative phosphorylation by *UL97* protein kinase in cytomegalovirus and related orthologues in the other β herpesviruses (Littler *et al.*, 1992; Sullivan *et al.*, 1992). The high degree of conservation among the herpesviruses in the DNA polymerase is very predictive for broad efficacy of foscarnet, cidofovir, and the 4-oxoquinoline derivatives. The exception is the reduced susceptibility of HHV6A,B, that can be explained by an unusual V823A polymorphism in the active site of these viruses. Of interest, both of the benzimidazole analogs (maribavir and BDCRB) exhibit a very limited spectrum of activity, which is not predicted given the well conserved molecular target, particularly with BDCRB. Like other computational methods described herein, their greatest utility of this approach is generating a hypothesis to be tested in the laboratory. Future mechanism of action studies for this series of compounds will be needed to explain the limited spectrum of activity for this series of compounds.

Conclusions

As genomics and related technologies develop, they will be applied to the study of herpesvirus biology and to the discovery of new antiviral drugs to treat these infections. Initial reports have provided indications that these strategies may be particularly useful in this family of viruses. These approaches hold promise and will likely make substantial contributions to the field as the technologies mature.

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68

Candidate anti-herpesviral drugs; mechanisms of action and resistance

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Research into the molecular biology of herpes replication in recent years has revealed novel targets for drug development (Fig. 68.1). The characterization and functional assay of these targets have been facilitated by advancements in gene expression, protein purification, proteonomics, bioinformatics, and efficient robotic screening technologies. The pipeline for new herpes drugs has been expanding as drug candidates have evolved more rapidly due to improvements in chemical synthesis (i.e., combinatorial and parallel synthesis methods), and with aids for drug design (X-ray crystallography, in silico computer modeling tools, as well as chemoinformatics). Many new herpes inhibitors have been reported, and most of these possess novel modes of actions. Several have entered clinical evaluation, with some later discontinued because of safety issues. This chapter will describe promising drug candidates in early development that appear to act at individual steps of the viral replication cycle, and focus on those that have the most potential for success (Table 68.1).

The chemotherapy of herpes infections was markedly advanced by the discovery of the first, highly selective antiherpetic agent, acyclovir (ACV, Zovirax®; [9-(2-hydroxyethoxymethyl)guanine]) (Elion *et al.*, 1977). Since the introduction of this agent, there has been only incremental progress in new drug approvals for the myriad of diseases caused by this family of diverse pathogens. The drugs approved since the introduction of ACV include valacyclovir (VACV, Valtrex®, the L-valine ester prodrug of ACV, penciclovir (PCV; [9-(4-hydroxy-3-hydroxymethylbutyl-1-yl) guanine]), a related nucleoside analogue with a similar basis for drug action against HSV and VZV, and its prodrug, famciclovir (FCV, Famvir®). More efficacious treatment of CMV disease was achieved with yet another guanosine analogue ganciclovir (GCV, Cymevene®, Cytovene®; 9-(1,3-dihydroxy- 2-propoxymethyl)guanine), and its recently approved prodrug, valganciclovir (Valcyte®; L-Valine, 2-[(2-amino-1,6-dihydro-6- oxo-9H-purin-9-yl)methoxy]-3-hydroxypropyl ester, monohydrochloride),. The treatment of ocular CMV infections was advanced by the antisense agent ISIS 2922 (formivirsen, Vitravene®). Broad-spectrum antiherpetics include the pyrophosphate analogue foscarnet (PFA, Foscavir®; trisodium phosphonoformate, phosphonoformic acid) and the nucleotide analogue cidofovir (CDV, HPMPC, Vistide®; (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine).

Although these agents have proven efficacious in the prophylaxis and treatment of herpes infections, there remains a need for drugs with higher potency, more rapid and durable antiviral action, more convenient dosing regimens, and importantly, fewer and less severe side effects. Because these systemic drugs ultimately target the viral DNA polymerase, and the nucleoside analogs are viral TK-dependent, cross-resistance can occur (Erice, 1999). New drugs with novel mechanisms of actions would provide valuable alternatives. Ideally, the new drug would eliminate the latent reservoir; a challenging goal since the herpesviruses have evolved complex strategies to persist under the reach of host defense mechanisms.

The drug development process: an overview

The scientific literature abounds with reports of the in vitro anti-herpetic activities of diverse organic molecules and biological products, and in many cases, the selectivity index (SI; the ratio of cellular toxicity to antiviral potency in vitro) appears promising (Snoeck *et al.*, 2002). However, the demonstration of in vitro activity is only the first step in the long and arduous journey from the laboratory bench into the clinic (Scolnick *et al.*, 2001). Other considerations, particularly absorption, distribution, metabolism and excretion (ADME) parameters are

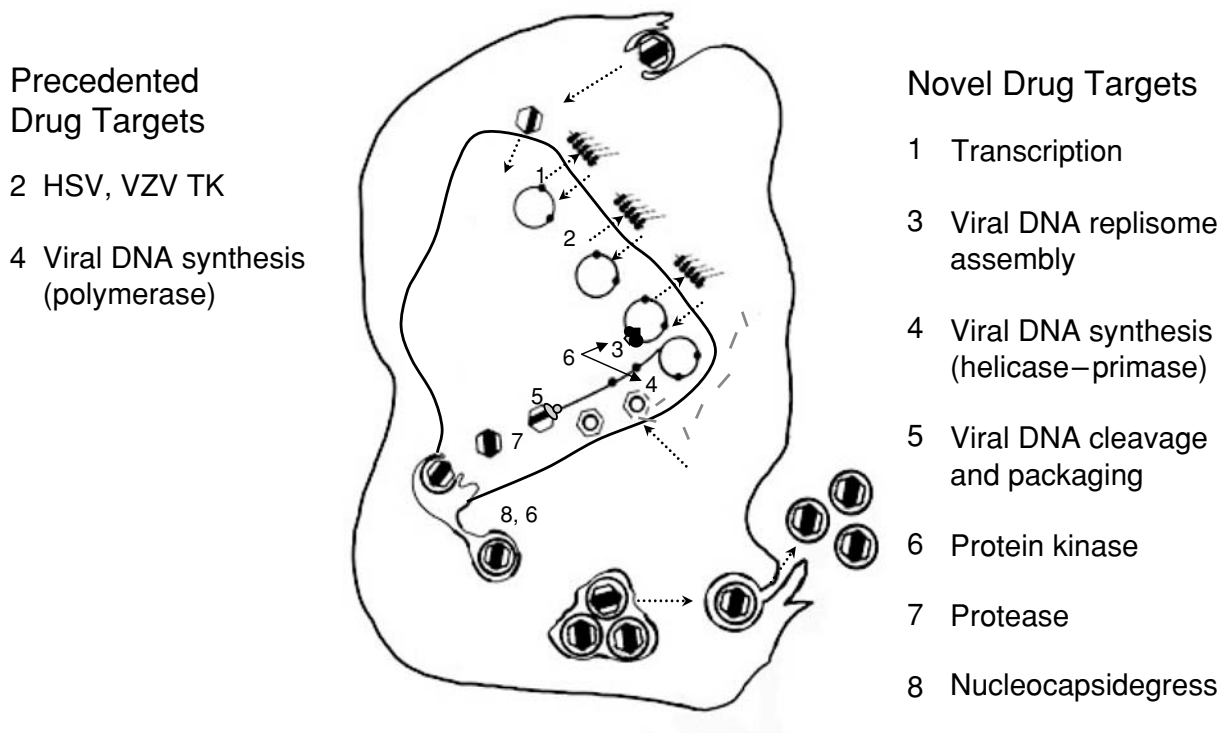


Fig. 68.1. Herpesvirus replication cycle illustrating precedented and novel drug targets (numbered) and their general stage of function in the viral replication cycle. (Adapted from Roizman *et al.*, 1993.)

key to success. Lipinski's rule-of-five analysis (Lipinski *et al.*, 2001) which established guidelines regarding structural properties most often associated with viable drug candidates has been embraced by the pharmaceutical industry.

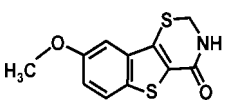
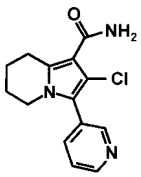
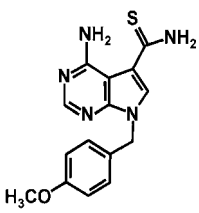
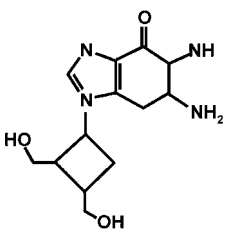
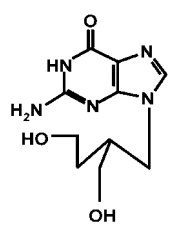
The discovery and development process is slow and costly. The industry average to bring a new drug to market was estimated at over $500 million dollars for drugs introduced in 1990 and is undoubtedly higher today (Boston Consulting; Pharmaceutical R&D Costs, 1993). The time from synthesis of a new drug to regulatory approval has grown to over 14 years from an average of 8 years in the 1960s, according to analyses by the Tufts Center for the Study of Drug Development. Figure 68.2 provides a schematic review of some of the major preclinical development activities generally required to advance a drug to approval.

Historically, anti-infectives have been discovered through the screening of compound libraries directly against the replicating organism. This classic approach was used in the discovery of all the currently approved systemic antiherpetics. ACV, which was originally synthesized to potentiate the anticancer activity of the nucleoside analogue, cytosine arabinoside (Ara C), by inhibition of adenosine deaminase (the enzyme responsible for its metabolic breakdown), emerged as a potent antiherpetic during random screening in herpes simplex virus (HSV)-infected cells (Elion *et al.*, 1977).

Screening of diverse chemical libraries in virally-infected cell cultures, although less efficient than individual enzyme or target-based screens (discussed below), provides the opportunity to identify new viral targets. Active inhibitors can then serve as laboratory tools to probe the biology of viral replicative events. The mechanisms of novel action are defined by identifying changes in the phenotype of infected, treated cells, often aided by time of inhibitor addition and withdrawal experiments. Antiviral selectivity is indicated by the ability of the virus to develop resistance to escape drug inhibition, and genetic mapping of resistance mutations identifies the viral target(s).

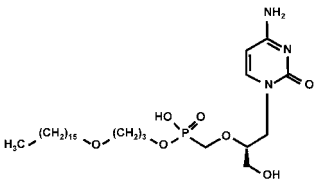
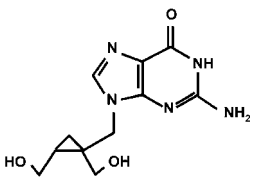
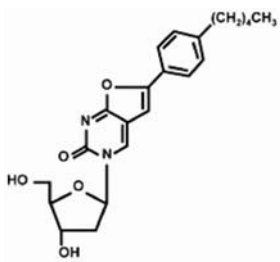
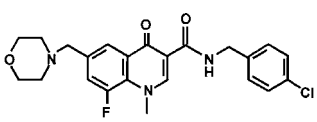
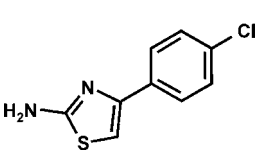
A second approach to the discovery of new inhibitors consists of direct screening of compounds against a catalytic enzyme or other biological function. Often screening and inhibitor optimization may be directed by structural

Table 68.1. New anti-herpes inhibitors in the discovery and development pipeline

Drug/ compound class/ chemical structure	Virus/ antiviral activity per status (early 2004)	Mechanism of action (MOA)/target gene(s)	Key reference(s)
Inhibitors of early replication events			
PD146626 (benzothiophene) 	• HSV-1, IC_{50} 0 ~ 0.1 μM • Cytotoxicity ≥1.0 μM (HFF) • early preclinical	• MOA unknown; MOI-dependent in vitro activity • blocks IE (VP16 and ICPO) gene expression • cell target likely no resistant virus	Boulware *et al.*, 2001 Hamilton *et al.*, 2002
CMV423 (tetrahydroindolizine derivative) 	• CMV, HHV-6, HHV-7 • IC_{50} range CMV:0.005–0.05 μM (at low MOI) • IC_{50} range HHV-6: 0.1–0.3 μM • activity cell-type dependent for HHV-6 • Preclinical	• MOA unknown;MOI-dependent in vitro activity • cell target likely: no resistant virus • host tyrosine kinase may be involved • blocks early event prior to viral DNA synthesis	Snoeck *et al.*, 2002 DeBolle *et al.*, 2004 Cirone *et al.*, 1996
828, 951, and 1028 non-nucleoside pyrrolo[2,3-*d*]pyrimidines  **951**	• CMV • all three compounds comparable to GCV (IC_{50} 0.4–1.0 μM) • early preclinical	• MOA unknown; MOI-dependent in vitro activity • blocks early event prior to viral DNA synthesis	Jacobson *et al.*, 1999
Inhibitors of herpes DNA polymerase			
Lobucavir (LBV) (cyclobutyl guanosine analogue) 	• VZV, CMV, HBV • clinical anti-CMV activity demonstrated in HIV-infected subjects • development terminated 2002.	• phosphorylated by cellular enzymes • triphosphate a potent inhibitor of CMV DNA polymerase (limits chain extension) • target gene: pol	Tenney *et al.*, 1997 Dunkle, 1996 *Lalezari et al., 1997*
Omaciclovir (H2G) Carbocyclic guanosine analogue Prodrug MIV-606  H2G	• VZV, HSV-1 and 2, EBV • More potent vs VZV than ACV • efficacy demonstrated in simian VZV infection in African green monkeys • resistance profile similar to ACV for TK- mutants • phase II clinical studies	• MOA similar to ACV: – substrate for VZV & HSV 1 & 2 TK – competitive inhibitor of viral DNA polymerase • MOA differences from ACV: – not an obligate chain terminator, but limits chain extension – substrate for mitochondrial TK – longer intracellular $t_{1/2}$ of H2G-TP • resistance mutations: – VZV TK frameshift mutations: – dels A76, G805, or A806 produce truncated polypeptide – cross resistant to ACV	Abele *et al.*, 1991 Soike *et al.*, 1993 Lowe *et al.*, 1995 Ng *et al.*, 2001

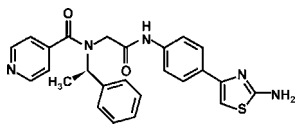
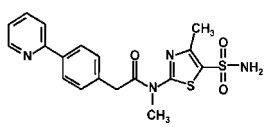
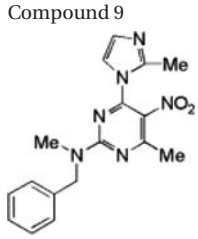
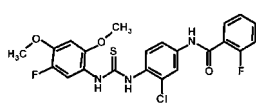
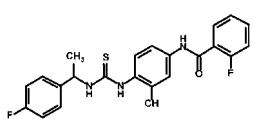
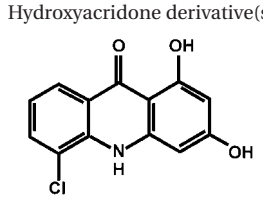
(*cont.*)

Table 68.1. (*cont.*)

Drug/ compound class/ chemical structure	Virus/ antiviral activity per status (early 2004)	Mechanism of action (MOA)/target gene(s)	Key reference(s)
Alkoxyalky ester of CDV (nucleotide analogue) 	• broad spectrum antiviral • herpes activity $IC_{.50.}$ range 0.5–30.0 μM • potential therapeutic for smallpox, rescue of herpes-resistant infections in immunocompromised • accelerated development through Phase I for smallpox	• nucleotide monophosphate, further phosphorylated by cell enzymes • competitive inhibitor of viral DNA synthesis • single point mutations in pol confer CDV-resistance	Ciesla *et al.*, 2003 De Clercq, 2002 Safrin *et al.*, 1997 Neyts *et al.*, 2004 Aldeon *et al.*, 2003 Painter and Hostetier, 2004 Kern *et al.*, 2004a
A-5021 Guanosine analogue 	• HSV-1 & 2; $IC_{.50.}$ range 0.013–0.15ug/ml • VZV IC50 ~ 0.77 ug/ml • more potent vs HSV-1 than ACV in vitro and in animal models	• MOA similar to ACV: – substrate for VZV & HSV 1 & 2 TK – competitive inhibitor of viral DNA polymerase – longer intracellular $t_{1/2.}$ of A-5021-TP vs ACV-TP – not a chain terminator, but limits chain extension • resistance profile similar to ACV for TK mutants	Ono *et al.*, 1998 Iwayama *et al.*, 1998 Iwayama *et al.*, 1999
BCNAs bicyclic pyrimidine nucleoside analogues 	• VZV • highly potent at sub-nanomolar concentrations IC50 1nM10–μM	• MOA not fully elucidated: – substrate for VZV TK – TK- deficient VZV also resistant to BCNAs – no triphosphate detected in VZV-infected cells	Balzarini and McGuigan, 2002 DeClercq, 2003a,b McGuigan *et al.*, 2003 Sienaert *et al.*, 2002 Balzanini *et al.*, 2002
PNU-183792 non-nucleoside (4-oxo-DHQs) naphthalene carboxamide 	• VZV, HSV, CMV, HHV8, EBV • CMV $IC_{.50.}$ ~ 0.95 μM; comparable to GCV • HSV-1 & 2,VZV, HHV8 IC50 ~3.5 μM • EBV IC_{50} 0.17 μM • no detectable cross-resistance with approved antivirals	• inhibitor of viral DNA polymerase • competitive inhibition with the binding of natural substrate (dTTP) to the polymerase enzyme, with a low level affinity for the enzyme substrate complex that was not defined	Vaillancourt *et al.*, 2000 Oien *et al.*, 2002 Thomsen *et al.*, 2003 Wathen, 2002
Inhibitors of herpes helicase-primase			
T157602 2-aminothiazole 	• HSV • $IC_{.90.}$ = 3 μM	• Inhibits viral DNA synthesis • stabilizes the helicase-primase complex trapping the enzyme on the DNA substrate, and blocking all activities of the complex.	Spector *et al.*, 1998

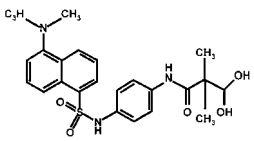
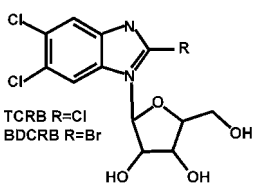
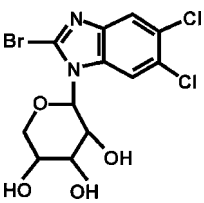
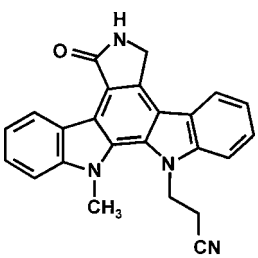
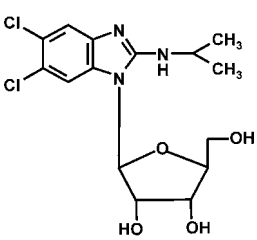
(*cont.*)

Table 68.1. (*cont.*)

Drug/ compound class/ chemical structure	Virus/ antiviral activity per status (early 2004)	Mechanism of action (MOA)/target gene(s)	Key reference(s)
BILS 179 BS thiazolylphenyl containing compounds 	• HSV • 10-15 times more active than ACV in vitro ($EC._{50s.}$ 27nM- 100nM) • Efficacy studies in animals demonstrated comparable &/or superior efficacy to ACV	• stabilizes the helicase-primase complex and DNA substrate, imposing a physical constraint both to enzyme progression (DNA-unwinding reaction), and primase catalytic activity • Resistance mutations in UL5 • Rapid selection of stable, pathogenic phenotype	Crute *et al.*, 2002 Kleyman *et al.*, 2003a,b Liuzzi *et al.*, 2004
BAY 57-1293 triazole urea compounds 	• HSV $IC._{50.}$ 20 nM • ~100-fold more sensitive that ACV against HSV	• inhibits viral DNA synthesis • novel mode of action inhibits the ATPase activity of the viral helicase-primase complex in a dose-dependent manner ($IC._{50.}$ of 30 nM). • Resistance mutations in UL5, UL52	Kleymann *et al.*, 2002 Kleymann, 2003a Betz *et al.*, 2002
Compound 9 	• CMV IC50 0.039 μM • Range of analogs 0.4⇒10 μM • Leads comparable to CDV, GCV in potency	• Inhibits viral DNA synthesis • Target; UL70 component of helicase-primase complex, based on co-IP. • Irreversible covalent binding proposed; no viral resistance genetics confirmation yet reported	Cushing *et al.*, 2006
Inhibitors of the portal protein of HSV/ viral DNA packaging			
WAY 150138 benzamide thiourea compounds 	• alpha herpes viruses	• inhibitor of viral DNA cleavage & packaging • target gene: HSV UL6 • resistance mutations UL6 – Glu121Asp, Ala618Val, Gln621Arg	van Zeijl *et al.*, 2000, Visalli and van Zeijl, 2003 Newcomb and Brown, 2002
Comp I N-methylbenzyl-N'-arylthiourea analogues 	• VZV ($IC._{50s.}$ 0.1 to 0.6 μM)	• VZV DNA cleavage and packaging is restricted via inhibition of the ORF54 gene • ORF54 resistance in 2 isolates due to changes in codons 324 & 408 in 1 isolate and codon Gly407Asp in another isolate	Visalli *et al.*, 2003 van Zeijl *et al.*, 2000 Akanitapichat and Bastow, 2002)
Hydroxyacridone derivative(s)  5-chloro-1,3-dihydroxyacridone	• HSV $IC._{50.}$ ~10 μM • hCMV $IC._{50.}$ <10 μM (multiple derivatives) • early preclinical	• MOA unknown, selectivity questionable • diverse phenotype in treated, HSV-infected cells; reduction in: – viral DNA packaging – production of B capsids – infectious virions	Akanitapichat and Bastow, 2002 Lowden and Bastow, 2003

(*cont.*)

Table 68.1. (*cont.*)

Drug/ compound class/ chemical structure	Virus/ antiviral activity per status (early 2004)	Mechanism of action (MOA)/target gene(s)	Key reference(s)
Inhibitors of the CMV terminase complex			
BAY 38-4766 	• CMV • IC $_{50}$ ~1 μM • Anti CMV activity comparable to GCV in hollow fiber mouse model	• novel, late-stage inhibition of UL89 and UL56 gene products (2 subunits of the CMV terminase) • reduction in viral DNA packaging • resistance mutations in UL89, UL56	Buerger *et al.*, 2001 Reefschlaeger *et al.*, 2001 McSharry *et al.*, 2001a,b
TCRB & BDCRB benzimidazole ribosides 	• CMV • IC$_{50}$ < 3 μM	• inhibits viral DNA cleavage and packaging • target genes: UL89, UL56 • resistance mutations: – pUL89: Asp344Glu, Ala355Thr – pUL56: Gln204Arg	Townsend *et al.*, 1995 Underwood *et al.*, 1998 Krosky *et al.*, 1998
GW275175X B-D-ribopyranosyl derivative of BDCRB 	• hCMV; IC$_{50}$ range 0.16-2.03 μM • antiviral activity similar to GCV in vitro • not active vs animal CMV; SCID hu efficacy model tested • Phase I single-dose study completed	• inhibits viral DNA cleavage and packaging • target genes UL89, UL56 • mutations conferring cross resistance: – UL89: Asp344Glu, Ala 355Thr UL56: Gln204Arg	Underwood *et al.*, 2003 Williams *et al.*, 2003 Krosky *et al.*, 1998
Inhibitors of the CMV UL97 protein kinase			
Indole carbazoles 	• hCMV; IC$_{50}$ range 0.009–0.4 μM	• inhibits pUL97autophosphorylation and pUL97-dependent GCV phosphorylation • target gene UL97	Zimmermann *et al.*, 2000 Marschall *et al.*, 2001 Slater *et al.*, 1999
Maribavir, 1263W94 	• CMV, IC$_{50}$ values are assay, cell-type dependent, and range from 0.08–26 μM • generally 3–10-fold more potent than GCV • EBV; IC$_{50}$ values for lytic viral DNA synthesis reduction 0.2–17 μM	• inhibits viral DNA synthesis and nucleocapsid maturation/egress • target genes/resistance mutations – UL97: Leu397Arg (AD169) – UL27 gene: Leu335Pro (Towne), Arg233Ser (AD169), Ala406Val, Cys415 stop (AD169), Trp362Arg (AD169)	Biron *et al.*, 2002 Komazin *et al.*, 2003 Chou *et al.*, 2004 Lalezari *et al.*, 2002 Williams *et al.*, 2003 Ma *et al.*, 2006

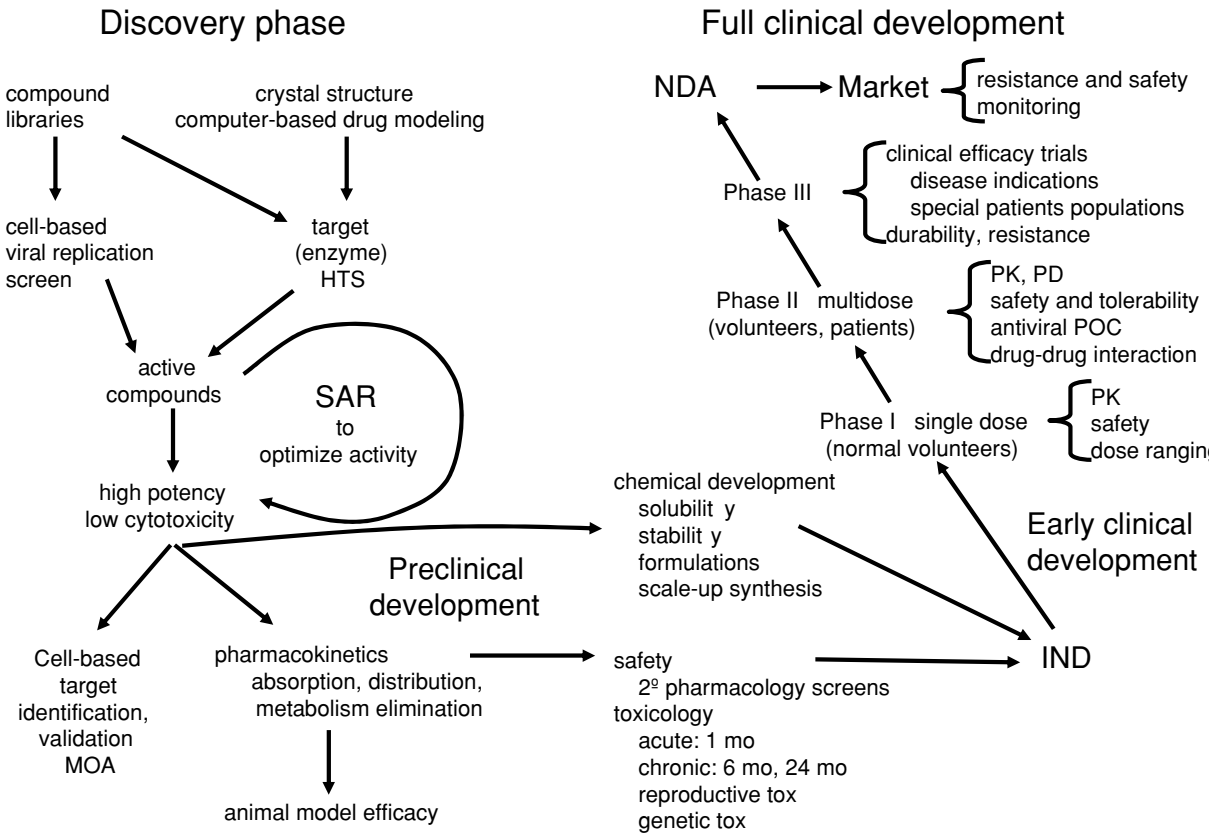


Fig. 68.2. Schematic overview of drug development process.

modeling. This approach is highly efficient, and has been particularly successful in the human immunodeficiency virus (HIV) arena, producing candidates or approved drugs targeting HIV entry, fusion, genetic integration, protease, and the HIV reverse transcriptase. Significant efforts to identify herpes protease inhibitors have not been successful to date, but direct screening of herpes DNA polymerases and the helicase-primases have produced exciting drug candidates with strong development potential.

A few principles emerge from the collective experiences in the field of antiviral drug discovery. The ideal target will be unique to the virus, or sufficiently distinct from the host cell counterparts to allow preferential inhibition, and will be essential for viral replication (in vitro for assay purposes) and for disease pathogenesis in the host. The genetic barrier to resistance will be high, and drug-resistant variants will pay a penalty in replication competence or tissue tropism. In the case of the alpha herpesviruses, drug-resistant variants will lack the ability to reactivate from latency.

An active antiviral compound must have other attributes in order to progress successfully through the drug development process. The chemical and pharmaceutical development criteria of cost-effective manufacturing, product stability, good solubility, oral bioavailability, and acceptable protein binding preclude development of high molecular weight compounds, or complex biological products, except as topical or injectable formulations. For the well-defined catalytic sites of many target enzymes, small molecule inhibitors ($\leq$600 Da MW) can provide the ideal "fit." However, for the less well-defined catalytic sites of some enzymes, or the broad surfaces of protein–protein interactions (Tsai *et al.*, 1997), larger MW inhibitors, such as protein mimetics, may be required. Protein-protein interactions comprise many opportunities for antiviral intervention, but from a pharmaceutical perspective, these remain challenging targets (Arkim and Wells, 2004).

Host cell targets as an approach to virus inhibition

Drugs that target host cellular functions have been considered as potential antiviral agents (Shugar, 1999) and the herpes virus literature documents the antiherpes activity

of a variety of host cell kinase inhibitors, such as roscovitin and inhibitors of p38 and cdk-E (Schang, 2002; Schang *et al.*, 2002; Chang, 2003). Investigators have looked at the effects of various antimetabolites on the ability of host cells to support viral replication. Although these are useful probes for discerning the contributions of host functions to viral growth, none have yet been shown to have clinical development potential as systemic therapies for the herpes diseases.

Reveratrol, a natural plant phytoalexin, was recently shown to inhibit HSV 1 and 2 replication early in the infection cycle by inhibition of ICP-4 (Docherty *et al.*, 1999). The mechanism may not directly involve a virus target, but could have application as a topical agent similar to the anti-inflammatory effect of n-docosanol 10% cream (Abreva®). One category of host targets which may be fruitful for the development of novel therapeutics that deserves mention here are immune response modulators. The herpes viruses have developed multiple ways to evade the host's innate immunity and to block antigen presentation required for the adaptive immune responses. Thus, agents which protect or augment host defense mechanisms by interfering with viral immune evasion functions may provide additional tools in disease management. Such agents could complement antiviral drugs and may potentiate vaccine efficacy (Miller *et al.*, 2002). For example, Imiquimod, an immune response modifier approved for the topical treatment of external genital and perianal warts, has recently been shown to be an agonist to the toll-like receptor 7 (TLR-7) (Hemmi *et al.*, 2002). The related analog, resiquimod, has also been shown to have immunomodulatory properties and efficacy as a topical therapeutic agent in genital HSV-2 infections models (Bernstein *et al.*, 2001; Miller *et al.*, 2002). TRL-7 may be involved in the induction of cytokines, especially alpha interferon, and other antiviral effector molecules which may enhance cell mediated immunity (Wang *et al.*, 2005). Moreover, Imiquimod has also been shown to have potent adjuvant/priming properties in several vaccine models (Rechtsteiner *et al.*, 2005; Thomsen *et al.*, 2004). Several candidate TLR agonists, plus other immunomodulatory agents are in development for various viral diseases, but will not be discussed further.

Antiviral targets in early replication events

In principle, an inhibitor that blocks the very earliest steps in the invasion of a cell by a virus should effectively restrict the spread of infection and could also serve as a prophylactic agent. However, inhibition of herpes viral attachment and uncoating may not be feasible, since there are no unique, restrictive mechanisms for herpes entry that could be exhaustively (and presumably safely) disabled. This is in contrast to the prospects for the antagonist of the CCR5 host receptor element required for HIV infection (Baba *et al.*, 1999), the recently approved HIV fusion inhibitor (LaBranchea *et al.*, 2001) in HIV infection, and the rhinovirus uncoating blocker (Diana *et al.*, 1987).

Immediate early gene (IE) expression or the transactivation functions of their products could after the earliest replication events for intervention, and their inhibition should effectively restrict productive viral infections and potentially decrease reactivation from latency. However, since the transcription of viral genes and the viral-mediated transactivation intimately involve host transcriptional machinery and factors, selectively would be difficult to attain.

Three or four series of compounds with designated early modes of action have emerged from cell-based screening programs. Time-of-addition studies or quantitation of viral transcription and translation indicated that these classes of compounds acted after the adsorption phase of HSV or CMV infection but before viral DNA synthesis. Two striking features of their mechanism of action (MOA) were common to these agents: their in vitro potency was compromised by increased multiplicity of infection (MOI), and the investigators were unable to select resistant viruses, despite repeated attempts to passage virus in the presence of the inhibitors. These features, particularly the latter, are consistent with inhibition of a cellular target. In the case of CMV423 (see below), the inhibition of a host protein kinase is demonstrated. Although the in vitro SIs for these agents indicate at least a preferential inhibition of viral growth, drug development potential of these compounds is doubtful. They will be briefly described.

PD146626

The activity of the benzothiophene class was identified in a random compound screening program. The lead compound, PD146626 (9-(methyloxy)-3,4-dihydro[1]benzothieno[2,3-f][1,4]thiazepin-5(2H)-one), was shown to inhibit HSV type 1 (HSV-1) replication by blocking immediate early viral gene expression, specifically VP16 and ICPO expression. However, viruses deleted for the VP16 and ICPO loci ("knock-out" viruses) lacked resistance to PD146626, and the compound showed apparent anti-viral activity against CMV, which lacks VP16 and ICP0 homologues. Moreover, PD146626 could exert this inhibitory effect in cells pretreated before viral infection, or in cells treated with only short exposures (up to 2 hours) during viral infection. Thus, PD146626 apparently targets a cellular function critical for the expression of HSV-1 immediate early genes (Boulware *et al.*, 2001). Extensive SAR studies that focused on stereospecific substitution on the diazepine ring and optimal nitrogen substitution achieved striking improvements as evidenced by a 2-log

enhancement in potency and a 3-log improvement in therapeutic index. However, in vivo efficacy could not be determined due to metabolic issues, and thus the safety consequences of this inhibitory mechanism remains to be determined (Hamilton *et al.*, 2002).

Non-nucleoside pyrrolopyrimidines 828, 951, and 1028
Three structurally distinct analogues in a series of non-nucleoside pyrrolo[2,3-d]pyrimidines emerged from a cell-based screening program (Jacobson *et al.*, 1999). At low MOIs compounds 828, 951, and 1028 all showed potency comparable to that of GCV. One of these compounds, 828, was tested for toxicity and shown to be less toxic against human bone marrow progenitor cells than GCV, a key improvement.

CMV423
Perhaps the best-studied agent with activity early in the herpes life cycle is CMV423, 2-chloro-3-pyridin-3-yl-5,6,7,8- tetrahydroindolizine-1-carboxamide. This tetrahydroindolizine derivative is active against CMV, HHV-6, and HHV-7 at low concentrations, but shows only modest activity against HSV-1 and -2 and none against varicella-zoster virus (VZV) (Snoeck *et al.*, 2002; De Bolle, 2004). The synergistic activity against CMV observed when CMV423 was combined with GCV, PFA, or CDV suggested that CMV423 was inhibiting a different step in viral replication, most likely an earlier one, than these DNA polymerase inhibitors. A series of studies aimed at defining the point of inhibition, using the low multiplicity of infection (MOI) multi-cycle format and probing for the expression of the IE (immediate early) and E (early) antigens showed transient reductions in the levels of IE antigen detectable on days 1 and again on days 4 and 5, in concert with first- and second- round of viral replication. Interestingly, CMV423 was able to block substantial expression of IE antigen at the viral input of 0.1 PFU/cell, an MOI at which antiviral activity is lost, indicating that low IE expression is sufficient to overcome the block to replication. This result suggests that any drug directed against the CMV IE gene would have to be almost 100% effective to produce the desired virus suppression.

Further work on the mechanism of inhibition of human herpesvirus (HHV)-6 replication again pointed to a cell target, as inhibition occurred in a cell-line dependent fashion (De Bolle *et al.*, 2004). The molecular target in HHV-6 is most likely a different early event, occurring before viral DNA synthesis but after IE antigen production. Based on the similarity of the action of herbimycin, which is known to inhibit cellular tyrosine kinase activity through binding to heat shock protein (Cirone *et al.*, 1996) and to block infection of human T lymphoid cells by HHV-6, the antiviral action of CMV423 is likely to be mediated through inhibition of a host cell tyrosine kinase. Preclinical safety and pharmacokinetic studies on this interesting inhibitor continue (Aventis, data on file; Bournique *et al.*, 2001). The clinical relevance of the MOI-dependence, and the safety margin with host inhibitory mechanisms, remain to be determined.

ISIS 13312
One very specific and clinically validated inhibitor of CMV IE gene expression is the approved anti-sense agent formivirsen (ISIS 2922, Vitravene®). Its utility is greatly limited due to the need for monthly intravitreal injections, and the occurrence of adverse ocular reactions. ISIS 13312, an analogue of ISIS 2922, has been shown to have a longer half-life than ISIS 2922 (approximately 2 months in monkey retina) and could provide the advantage of less frequent dosing (Henry *et al.*, 2001). However, ISIS 13312 is not currently in clinical development.

Antiviral targets in the herpesvirus DNA replication complex

The six or seven essential proteins that comprise the herpes viral DNA replication machinery offer several attractive enzyme targets for drug development, as well as some of the more challenging protein-protein interactions (Matthews *et al.*, 1993; Anders and McCue, 1996; Loregian and Coen, 2006). These components of the HSV replication machinery include the single-stranded DNA binding protein (ICP8, pUL29), the polymerase accessory factor (pUL42), the helicase-primase complex (pUL5, pUL8, pUL 52) and the viral DNA polymerase (pUL30). HSV requires an origin-binding protein specifically (pUL9). These proteins work in concert, are co-localized within specific intranuclear structures, and are found in association with other viral and host proteins involved in the replication cycle events. In principle, points of antiviral intervention could include those that affect protein recruitment and transport (post-translational modification), the sequentially–ordered protein–protein binding events in replisome assembly, and the individual catalytic functions of the enzymes involved in the DNA synthetic process.

The direct inhibition of the DNA polymerase function through nucleoside/nucleotide substrate analogues or pyrophosphate mimics, such as seen with the ACV, GCV, and PFA, is a clinically-validated approach. Discovery efforts continue to exploit this well-validated target, usually in concert with the viral encoded nucleoside kinase for added selectivity in the monophosphorylation step (or also including thymidylate kinase activity). Successful

inhibition of the HIV RT through non-catalytic mechanisms (non-nucleotide reverse transcriptase inhibitors such as nevarapine and efaverenz) have prompted the search for similar agents in herpes discovery screening programs. Representatives in both these categories are in advanced preclinical or early clinical testing. Also in the pipeline are agents that interfere with the function of the helicase primase complex (Fig. 68.3).

Herpesvirus DNA polymerase inhibitors

The herpes virus DNA polymerase is a multifunctional enzyme that possesses both a deoxynucleotide polymerizing activity and a 3′–5′ exonuclease proofreading function, and the structure of the herpes virus replicating complex has been modeled (Franklin *et al.*, 2001). The polymerase polypeptide shares regions of sequence similarity with the catalytic subunits of other alpha – like DNA polymerases of eukaryotes (Braithwaite and Ito, 1991). The conserved regions involved in substrate recognition within the polymerase have been determined by comparative modeling with the Klenow polymerase, and by genetic analysis of mutants resistant to nucleoside/nucleotide analogs (Gibbs *et al.*, 1998; Larder *et al.*, 1987). These regions (I, II, III, V, VII and the delta *region C) are non-contiguous, indicating the broad areas of contact across the polymerase polypeptide during the catalytic polymerization process. The ability of an inhibitor to interfere with correct folding through binding outside of the catalytic sites could impair enzyme function, although mutational escape may be more feasible, based on precedence in the HIV NNRTI series (Spence *et al.*, 1995).

Nucleoside/nucleotide analogue inhibitors

The apparent tolerance of the herpes DNA polymerases for modified acyclic and carbocyclic sugar moieties, exemplified by ACV, GCV, and PCV, drove additional exploration in the purine nucleoside series during the late 1980s and the early 1990s. The discovery of oxetanocins with the structural characteristic of two hydroxymethyl groups located on a rigid 4-membered ring led to the synthesis and antiviral evaluation of a number of related compounds and investigation of their antiviral properties (Sakuma *et al.*, 1991; Sekiyama *et al.*, 1998). Compounds in the oxytanocin series of base analogues, characterized by a carbocyclic sugar moiety, were investigated and showed broad-spectrum activity against the herpesviruses.

Lobucavir

Lobucavir, (R)-9-[4-hydroxy-2-(hydroxymethyl)butyl] guanine], (LBV, cygalovir, BMS 180194), a cyclobutyl analogue of guanine arose from the oxytanocin series and was advanced through early clinical evaluations (Yang *et al.*, 1996a, b). The ultimate outcome serves to illustrate the risks associated with the discovery and development process.

LBV has antiviral activity against HIV, hepatitis B virus, and most herpesviruses, and the triphosphate of LBV is a potent inhibitor of hCMV DNA polymerase in vitro. However, Tenney *et al.* (1997) showed that this nucleoside analogue is phosphorylated intracellularly to its triphosphate form in both infected and uninfected cells, with phosphorylated metabolite levels only two- to three-fold higher in CMV-infected cells compared to uninfected cells. The lack of selective anabolism in virally infected cells (a factor contributing to the broad antiviral activity) provides the potential for substrate utilization by host cell DNA polymerases with corresponding safety risks.

LBV was advanced to the clinic. Preliminary human data showed a dose-related anti-CMV effect (Dunkle, 1996). A clinical study in HIV- and CMV-co-infected patients demonstrated a 50% reduction in CMV viruria and a greater than 1 log reduction in HIV viral load from semen at the highest dose. Side effects were dose-related, and included mild to moderate diarrhea and nausea in 10%–20%, and 7%–12% of recipients respectively (Lalezari *et al.*, 1997).

Despite promising early clinical results, an international Phase III study of LBV as therapy for hepatitis B was suspended in February 1999 due to safety concerns. Toxicologic studies in rodents had suggested increased incidence of stomach, vaginal and cervical cancers with long-term exposure.

Omaciclovir H2G and its pro-drug

Another carbocyclic guanosine analogue, H2G, (*R*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (omaciclovir), was shown to be a potent broad-spectrum antiherpes agent especially active against VZV (Abele *et al.*, 1991). The MOA is similar to that of ACV, with less selectivity as a substrate for TK. Resistance mechanisms at the TK locus overlap with those of ACV (Ng *et al.*, 2001). H2G is not an obligate chain terminator, although once incorporated, H2G-MP will only support limited chain elongation (Lowe *et al.*, 1995). The triphosphate of H2G has a considerably longer intracellular half-life in infected cells than does ACV-triphosphate (Lowe *et al.*, 1995), a feature that could provide dosing advantages over VACV if clinically validated. Preclinical efficacy studies in the simian varicella model indicated superior potency over ACV (Soike *et al.*, 1993). However, species-specific differences in metabolism of ACV make such comparisons misleading. ACV oral bioavailability is lower in monkeys than in humans, and

the higher aldehyde oxidase levels in monkeys result in faster metabolic clearance in monkeys than in humans, dogs, and rodents (de Miranda and Burnette 1994; de Miranda and Good, 1992).

MIV-606

(ABT-606; [L-valine-(2-hydroxy-4-hydroxymethyl-butylyl) guanine]) is a prodrug of H2G that significantly enhances its oral bioavailability. MIV-606 is quickly converted to H2G, with undetectable concentration of parent prodrug MIV-606 (Medivir AB, Huddinge Sweden, unpublished data). – Three phase I studies with a total of more than 100 volunteers, including subjects 65 years of age and older, demonstrated that MIV-606 was safe and well tolerated after multiple dosing up to total daily doses of 1500 mg.

A phase II study comparing 250, 500, and 750 mg twice daily of MIV-606 with 800 mg five times a day of ACV in zoster patients has also been completed. Trial results suggested equivalent or superior efficacy of MIV-606, compared to ACV, at significantly lower doses. If this claim can translate into an improved therapeutic effect of MIV-606 at a more convenient dose, it could provide enough improvement over current therapies to justify further development. Availability of MIV-606 could potentially lead to much wider treatment of zoster, and the broad spectrum of action could benefit patients with compromised immune function, such as transplant recipients, cancer patients, and AIDS patients (Medivir AB, Huddinge Sweden, unpublished data).

Alkoxyalkyl esters of cidofovir (CDV)

CDV is a nucleotide (monophosphate) analog with broad spectrum anti-herpes activity that is licensed for the intravenous treatment of CMV retinitis in HIV-infected patients. CDV is phosphorylated by cellular enzymes, and the CDV-diphosphate (DP) is a competitive inhibitor of viral DNA polymerase (Safrin *et al.*, 1997). Mechanistically, CDV-DP inhibits many viral DNA polymerases, and recent studies document activity against pox viral infections (Neyts *et al.*, 2004; De Clercq, 2002). In CMV, resistance to CDV can arise from single point mutations in the polymerase locus, usually mapping to the exonuclease functional domains (Chou *et al.*, 2003).

CDV exhibits a number of drawbacks that greatly limit its utility as an anti-herpetic agent. Oral bioavailability is low (<5%) requiring IV administration, usually on a weekly or semi-weekly basis, and dose-dependent nephrology may require pre-hydration, dose reduction and/or co-treatment with probenecid. Other safety liabilities were documented in preclinical toxicology studies Vistide® [package insert], Gilead Sciences, 1999.

Prodrug strategies, accelerated by the threats of bioterrorism, have been employed to increase oral bioavailability and improve the safety profile of CDV (Huggins *et al.*, 2002). The basic prodrug design exploited a natural fatty acid (lysophosphatidylcholine) molecule as carrier to facilitate drug absorption in the gastrointestinal tract. The lipid ester conjugates were much more active in vitro (EC_{50} values at least 100-fold lower) than CDV or cyclic CDV against a range of herpes viruses, including strains of HSV, VZV, CMV, EBV, HHV-6, and HHV-8. SAR of the ether lipid ester analogs defined a 20 atom optimum for alkyl chain length, and explored the nature of the linker group (Williams-Aziz *et al.*, 2005). Consistent with the observed increase in antiviral potency of the 1-O-hexadecyloxypropyl conjugate of CDV in cell culture, studies with radiolabelled compound confirmed increased cell penetration (10–20 fold) and higher intracellular levels (100-fold) of the active antiviral form CDV-DP than those measured in cells treated with CDV parent drug (Aldern *et al.*, 2003).

These lipid carrier prodrugs showed significant advantages over CDV in several in vivo models of murine and human CMV (Bidanset *et al.*, 2004; Kern *et al.*, 2004a; Wan *et al.*, 2005; Kern *et al.*, 2004b). Higher levels of protection were also achieved with the CDV oral prodrugs in a lethal cowpox challenge model (Huggins, 2002). Studies evaluating the oral bioavailability and tissue distribution of ^{14}C-labeled hexadecyloxypropyl-cidofovir (HDP-CDV), octadecyloxyethyl-cidofovir (ODP-CDV), and oleyloxypropyl-cidofovir (OLP-CDV) in female NIH Swiss mice demonstrated that these alkoxyalkyl esters are highly orally bioavailable (88–97%) and do not concentrate in the kidney (Ciesla *et al.*, 2003). Thus these compounds may avoid the dose-limiting toxicity of CDV, if these results translate into the clinic.

The lead compound, CMX001, will be progressed through phase I safety and pharmacokinetic studies in humans for potential use as a smallpox treatment or vaccine rescue (Painter and Hostetler, 2004). Such a therapeutic could also provide a safer salvage therapy for ACV or GCV resistant viruses in immunocompromised patients with life-threatening herpes infections.

This prodrug strategy was successfully applied to another nucleotide phosphonate 9-(S)-(3-Hydroxy-2-phosphonomethoxypropyl)adenine [(S)-HPMPA] with a similar enhancement of in vitro antiviral potency (Beadle *et al.*, 2006).

A-5021

Armed with structure activity relationship (SAR) clues from the crystal structure of the HSV-1 TK complexed with GCV (Brown *et al.*, 1995), and substrate potency

comparisons with ACV, PCV, H2G and the oxytanocins, the scientists at Ajinomoto set out to design a novel series of nucleoside analogues. Extensive exploration of the side chain conformation and enantiomeric specificity in the oxytanocin series lead to the identification of potent activity in a compound with a cyclopropyl sugar (Sekiyama *et al.*, 1998). The lead molecule in this series, **A-5021**, (1′S,2′R)-9-{[1′,2′-bis(hydroxymethyl)cycloprop-1′-yl]methyl}guanine, showed superior in vitro potency over the gold standards ACV or PCV against HSV-1 and VZV in vitro; however, the difference for HSV-2 was only marginal (Iwayama *et al.*, 1998). The compound was also active against EBV and HHV-6, but not HHV-8 (Neyts *et al.*, 2001). Since HSV-2 infection remains the most prevalent disease worldwide, A-5021 must show other development advantages over ACV, VACV and FCV to warrant the time and development investment for the genital herpes indications.

The mechanism of action of A-5021 was investigated (Ono *et al.*, 1998) and found to be qualitatively similar to that of ACV and PCV. A-5021 is anabolized to the monophosphate by the herpes TK enzymes and to the diphosphate by GMP kinase, as is ACV-MP. Levels of A-5021 triphosphate accumulating in HSV-1 or VZV-infected cells were higher than those for ACV-TP, but were roughly comparable to PCV-TP levels. The intracellular half life of A-5021-TP was longer than that of ACV-TP, but somewhat shorter than that of PCV-TP. However, ACV-TP had the most potency at the level of HSV DNA polymerase inhibition, with A-5021-TP intermediate in potency. Incorporation studies showed A-5201-MP could be incorporated into a growing DNA chain, although subsequent chain elongation was inefficient. The anti-HSV-1 and -2 activities were shown to be potentiated in vitro by the immunosuppressive agent mycophenolate mofetil, a finding consistent with the mechanism of competitive inhibition of GTP incorporation, since this agent is known to cause a reduction in cell dGTP pools (Neyts and De Clercq, 2001). A disadvantage of A-5021 is the likely cross-resistance with the most prevalent phenotype of ACV-resistant HSV, the TK-deficient phenotype.

A series of in vitro and in vivo studies suggested potential advantages of A-5021 over ACV for infections mediated by HSV-1. A-5021 exhibited more prolonged antiviral action than did ACV after short exposure of infected cells in vitro. This superior potency and durability carried over into several animal models of HSV-1 infection (Iwayama *et al.*, 1999). In a comparison using once-a-day oral administration with equivalent 25 mg doses, A-5021 demonstrated advantages over ACV in reducing the severity in HSV-1 cutaneous lesions. While the oral bioavailability and AUC of A-5021 is approximately half that of ACV, the superior in vitro potency and the prolonged effect contributed to better efficacy in this cutaneous HSV-1 murine infection model. When initiation of therapy by the intravenous route (100 mg/kg) was delayed to day 4 postinfection, A-5021 again was more effective in diminishing disease spread than ACV or PCV.

A-5021 treatment also resulted in a complete survival of mice infected intracerebrally with HSV-1 after IV dosing with 25 mg/kg A-5021 TID, compared to only 50% survival at 100 mg/kg IV ACV TID dosing (Iwayama *et al.*, 1999). The levels of A-5021 in the brain were not presented. Higher uptake of antiviral agent into the infected organ could be a major factor in the superior efficacy of A-5021 in this model, and a clear advantage of A-5021 over ACV, which has limited ability to penetrate the blood-brain barrier (de Miranda and Good, 1982).

In another variation of time of addition and withdrawal treatments in the animal model, high dose intrapertioneal (ip) infection of SCID mice was followed by once daily subcutaneous treatment (50 mg/kg). After 4 days of treatment, initiated at 1 hour, or 1 or 2 days post-infection, the delay in mortality and ultimate number of survivors was far greater in the A-5021 treated groups than in the ACV-treated groups (Neyts *et al.*, 2001). No pharmacokinetic information was provided to allow comparisons of actual systemic exposures.

In contrast to the superior performance of A-5021 against the gold standard of ACV in all the HSV-1 infection models, the efficacy of A-5021 was not distinguished from that of PCV in a model of systemic infection with HSV-2 (Iwayama *et al.*, 1999).

A-5021 has entered clinical development. It remains to be seen if the advantages in potency and duration of antiviral effects seen in cell culture and animal models will translate into superior efficacy in the various HSV and VZV disease indications. The ophthalmic use for herpetic keratitis is under development. Another proposed clinical application would be its use in gene therapy approaches to cancer, utilizing the HSV-1 TK vectors, since A-5021 is less cytotoxic than GCV, which is currently used (Hasegawa *et al.*, 2000).

BCNA compounds

A new structural class of bicyclic furo pyrimidines (BCNAs) have recently been discovered that demonstrate both highly specific and selective anti-VZV in vitro activity (Balzarini and McGuigan, 2002; McGuigan *et al.*, 2003; De Clercq, 2003a,b). The starting point for these compounds was BVDU, ((E)-5-(2-bromovinyl)-2′-deoxyuridine), which was established in the early 1980s as having good antivi-

ral activity but low selectivity. The BCNAs are characterized by a long alkyl or alkylaryl side-chain at the base moiety that may be responsible for both their antiviral properties and their lipophilic properties. The compounds are highly potent at sub-nanomolar concentrations, and cytotoxicity has not been observed at high micromolar concentrations.

The MOA has not been fully elucidated, but the compounds lose their antiviral activity against TK-deficient VZV strains, demonstrating that phosphorylation by the VZV-encoded TK is essential. Kinetic studies with purified enzymes revealed that the compounds were indeed a substrate for VZV TK, which is able to phosphorylate the BCNA compounds to both their corresponding 5′-mono and -diphosphate derivatives; a factor in their anti-VZV selectivity. Another indication of the unusual selectivity of this class of nucleoside analogs was the lack of substrate recognition by cellular kinases which contribute to the anabolism of other pyrimidine analogs; the cytosolic or mitochondrial TKs, cyrosolic dTMP kinase, and most striking, nucleoside diphosphate kinase, the host enzyme which converts BVDU-DP to the active triphosphate form. Consistent with this observation, no 5-triphosphate of BCNA could be detected in VZV-infected cells (Sienaert *et al.*, 2002). Information on the inhibitory effects of BCNA anabolites on the VZV DNA polymerase is not yet available.

There is no clear cut correlation between their affinity for VZV TK and the antiviral potency of the compounds, indicating that an additional SAR is likely (Balzarini and McGuigan, 2002). The closely related Simian varicella virus (SVV) is not sensitive to BCNA although in vitro studies indicate that SVV TK is able to phosphorylate BCNAs. Unfortunately this precludes the utility of the SVV animal model in the therapeutic development of BCNAs (Sienaert *et al.*, 2004).

The BCNAs are highly stable and not liable to breakdown by nucleoside/nucleobase catabolic enzymes (Balzarini *et al.*, 2002). The fact that they are not susceptible to degradation by thymidine phosphorylase and that they do not inhibit dihydropyrimidine dehydrogenase are key improvements over BVDU. Further clinical development is anticipated.

Non-nucleotide inhibitors

PNU-26730

In an effort to identify non-substrate inhibitors of the herpes polymerase with broad-spectrum activity, Pharmacia researchers set up herpes polymerase-based screens and tested 80 000 representatives of different compound diversity. Selectivity was achieved by secondary evaluation of hits against the mammalian DNA polymerases alpha, gamma and delta. The systematic discovery program identified the activity of the naphthalene carboxamide series, exemplified by the initial active compound, PNU-26730 (Vaillancourt *et al.*, 2000). Further optimization in this series led to a quinolone ring substitution and the 4-hydroxyquinoline-3-carboxamides series. Increased potency against the CMV, HSV-1 and VZV DNA polymerases was achieved by adding substitutions at the 6 position on the quinolone ring yielding the 4-oxo-dihydroquinolines (4-oxo-DHQs) series of compounds. These compounds, represented by PNU-182171 and PNU-183792, were also more active than the initial lead and the gold standard ACV against VZV and CMV, but showed no enhanced activity against HSV-1 or 2. These 4-oxo-DHQs were not active against other RNA and DNA virus tested: vaccinia, SV-40, adenovirus, HBV, influenza A, coxsackie B or VSV. (Brideau *et al.*, 2002; Knechtel *et al.*, 2002; Wathen, 2002).

PNU-183792

One of the 4-oxo-DHQ compounds, PNU-183792 (*N*-(4-cholorobenzyl)-1-methyl-6-(4-morpholinylmethyl)-4-oxo-1,4 dihydro-3-quinolinecarboxamide) was selected for additional investigations. Efficacy studies in a murine model of lethal MCMV infection showed antiviral activity similar to GCV when treatment was initiated up to 24 hours post infection, but was less efficacious at comparable doses given 48 hours postinfection (Brideau *et al.*, 2002). Other properties required for a drug candidate were demonstrated: for example, PNU-183792 was orally bioavailable in dogs and rodents, achieving concentrations above the IC_{50}, with reasonable rates of clearance and a half life of 3 hours in dogs. The important measure of available drug levels is at the intracellular site of action, and although this information was not published for PNU-183792, it is likely to be similar to the actual plasma concentrations. Nucleoside and nucleotide analogues may have an advantage over non-nucleoside inhibitors of DNA polymerase in this regard, as the $t_{1/2}$ of the triphosphate active form anabolized within the cell compartment can exceed plasma levels of unchanged drug.

The strong correlation between polymerase inhibition and viral replication inhibition in the analogue series supports inhibition of viral DNA polymerase as the MOA (Fig. 68.3). Further mechanistic studies into the nature of the polymerase inhibition showed competition with the binding of natural substrate (dTTP) to the polymerase enzyme, with a low-level affinity for the enzyme substrate complex that was not defined. Points of drug interaction with target protein were characterized by resistant virus

bearing point mutation(s) in the DNA polymerase gene (Oien *et al.*, 2002; Thomsen *et al.*, 2003).

This promising class of compounds will be active against the drug-resistant TK variants of HSV and VZV, and the current in vitro profile shows activity against clinically relevant HSV and CMV polymerase mutants (Thomsen *et al.*, 2003). This profile is consistent with this class of compounds interacting at a different molecular site in the polymerase polypeptide.

Information on the current development status is limited since Pharmacia was purchased by Pfizer. The selectivity screens have reduced the likelihood of mechanism-based toxicity within the series leads; and the safety profile will be defined by full in vivo toxiologic studies. Other key questions with this type of molecule and the nature of the MOA will be potency compared to valacyclovir or famciclovir, durability of action (related to frequency of dosing), and the ease of viral escape (resistance).

Herpes helicase-primase inhibitors

Efforts to identify drugs targeting other components of the DNA replication complex have focused on another enzyme, the helicase-primase (Hall and Matson, 1999). In the herpesviruses, the helicase-primase complex consists of 3 proteins that associate as a trimeric complex to carry out the essential tasks of unwinding the dsDNA in the 5′ to 3′ direction, RNA polymerase activity and ssDNA-stimulated ATPase activities (Crute and Lehman, 1991; Parry *et al.*, 1993). In HSV, these are the gene products of UL5, UL8, and UL52 ORFs. The drug candidates recently identified from screens targeting helicase-primase represent the next generation, and an exciting new class of antiviral agents. The major unknowns with this MOA include the ease of emergence of resistance in the clinic, and the pathogenicity (including reactivation) and transmission of potential of resistant virus.

T157602

The scientists at Tularik Inc. screened a library of >190,000 samples consisting of small organic molecules and natural products, using a novel filtration assay for the detection of the helicase DNA unwinding activity (Sivaraja *et al.*, 1998). The most active selective compound was a 2-aminothiazole compound, T157602 (Spector *et al.*, 1998). Preliminary MOA studies suggested that T157602 stabilized the helicase-primase complex, effectively trapping the enzyme on the DNA substrate and blocking all 3 activities of the complex (Fig. 68.3). The compound was a reversible inhibitor ($IC_{50} = 5\ \mu M$), of the helicase activity of the HSV UL5/8/52 complex, but was less active against other helicases, and could also interfere with primase activity at higher concentrations ($IC_{50} = 20\ \mu M$).

Strains of HSV-1 and HSV-2 resistant to T157602 that were selected in the laboratory carried individual point mutations in the UL5 viral gene that resulted in amino acid substitutions in the corresponding UL5 protein. Marker transfer studies confirmed the role of these genetic changes in the resistance phenotype, both at the level of the UL5 enzyme subunit and of mutant virus. Animal-model efficacy studies were not reported for this series.

In vitro cytotoxic studies revealed no apparent cellular toxicities at concentrations exceeding 100 μM, indicating a therapeutic window greater than 30-fold. However, as of 2003, the 2-aminothiazole compounds are no longer included in the Tularik development pipeline, suggesting safety deficiencies arose during the phase I/II clinical studies.

BILS 179 BS

Related compounds with more potent helicase-primase activity emerged from enzyme-based screens at Boehringer Ingelheim (Crute *et al.*, 2002) and cell-based viral replication screens at Bayer AG (Kleymann *et al.*, 2002). The thiazolylphenyl-containing compounds represented by BILS 179 BS inhibited all three enzyme activities of the HSV helicase-primase complex at 100 nM or lower concentrations. The antiviral activity was specific for HSV, with no activity against VZV, and human or murine CMV. Preliminary data suggest that the mechanism of inhibition involves a stabilization of the interaction between the enzyme complex and the DNA substrate, most likely by imposing a physical constraint both to enzyme progression through the DNA-unwinding reaction and to primase catalytic activity (Fig. 68.3).

Resistant viruses were selected by serial passage in BILS 179 BS for more definitive MOA studies. Helicase-primase purified from cells infected with these resistant viruses demonstrated decreased inhibition in an in vitro DNA-dependent ATPase assay that corresponded with antiviral activity. Single base pair mutations clustered in the N-terminus of the UL5 gene that resulted in single amino acid changes in the UL5 protein were identified by marker transfer and DNA sequence analysis. These results were consistent with helicase-primase inhibitor activity mediated through specific interaction with the UL5 protein (Liuzzi *et al.*, 2004).

BILS 179 BS was 10–15 times more active than ACV in vitro (EC_{50}s 27 nM–100 nM). Cytotoxicity effects were somewhat dependent on cell type, and additional experiments are needed to better clarify the cytotoxicity profile of these compounds. Efficacy studies were conducted

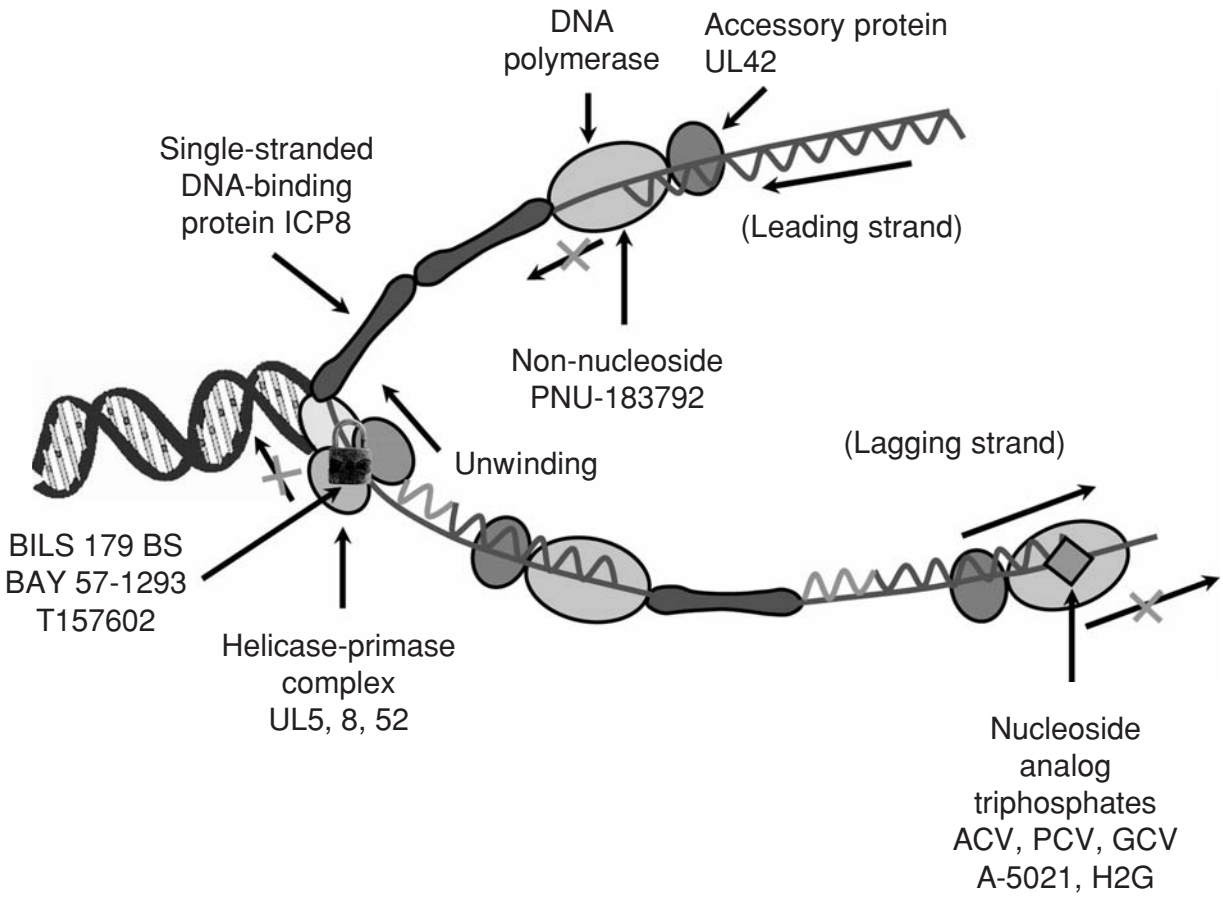


Fig. 68.3. HSV DNA replication targets. (Adapted from Crumpacker and Schaffer, 2002.)

in murine models of primary cutaneous and genital disease, using ACV as the treatment comparator (Crute *et al.*, 2002). In the cutaneous model of progressive zosteriform disease (hairless SKH-1 mouse infected with HSV-1 strain KOS), BILS 179 BS demonstrated comparable efficacy to ACV when treatment was initiated 3 hours post infection (1 × or 4 × daily). However, BILS 179 BS was superior to ACV when treatment frequency was reduced, or when initial treatment was delayed by 65 hours.

Comparable results were evident in the genital disease model (Swiss Webster mice vaginally infected with HSV-2 strain HG-52). Efficacy was based on a composite disease severity scoring system that included mortality. Again, BILS 179 BS had similar efficacy to ACV when treatment was initiated at 3 hours (1 × or 4 × daily), and superior efficacy when treatment frequency was reduced or when initial treatment was delayed by 65 hours. Actual drug exposures were not reported for these studies; however, BILS 179 BS was reportedly bioavailable, and on a weight-dosing basis, BILS 179 BS showed superior activity to ACV. Disappointing findings from a drug development perspective included the identification of pre-existing resistant variants within the wild type virus population, and rapid selection of highly resistant growth-competent viruses in vitro that maintained a stable drug-resistant phenotype in the absence of drug. Of more clinical relevance was the demonstration that two resistant strains studied were fully competent for disease pathogenesis (by cutaneous or intracerebral routes in mice), and for reactivation from latency in an ocular infection model (Liuzzi *et al.*, 2004). By contrast, ACV-resistant strains of HSV were generally less virulent in various infection models. This safety feature is based on the biology of HSV, and the mechanism of action of the drug ((Elion *et al.*, 1977). ACV resistant clinical isolates are generally TK-deficient, and as a consequence less pathogenic in the immune competent population and less competent for reactivation from latency (recurrences are ACV-susceptible). The potential for transmission of drug-resistant strains into the general population is a public health consideration and requires

careful monitoring (Shin *et al.*, 2001; Bacon *et al.*, 2003). The clinical development timetable for this compound is unknown at this time (mid 2006).

BAY 57-1293

The Bayer discovery program used a fluorometric high-throughput screening assay that identified inhibition in any target essential for viral replication in cell culture. Over 400,000 compounds were tested at 10 μM, and several compound classes with activity were identified (Kleymann, 2003 a,b). The triazole urea class of analogues was selected for additional study. Profiling and modeling techniques were employed, and optimization to improve the solubility led to the 2-pyridyl substituent in the para position of the phenyl ring in the lead compound to create BAY 57-1293 (*N*-[5-(aminosulfonyl)-4-methyl-1,3-thiazol-2-yl]-N-methyl-2-[4-(2-pyridinyl)phenyl]acetamide). BAY 57-1293 inhibited the replication of HSV type 1 and 2 in Vero cells (IC_{50} of 20nM) with a selectivity index of 2,500; ACV had an IC_{50} of 1 μM and a selectivity index of 250 under comparable conditions. There was no appreciable activity against VZV or CMV. BAY 57-1293 was equally active against ACV-resistant TK or polymerase mutants, and the activity was irrespective of cell type (Kleymann *et al.*, 2002). The in vitro replication block was reversible upon drug removal, and significant activity was still demonstrated when added late post infection.

Researchers selected virus resistant to each of the three analogues in the series. Resistance resulted from point mutations in UL5 (present in all six resistant strains), or in one case, a UL5 mutation together with a point mutation in UL52. These mutations in UL5 were clustered between nucleotides 1045 to 1077, a region that corresponds to the amino acids from 349 to 359 involved in the alpha helicase region that is most homologous across the herpesviridae (Kleymann *et al.*, 2002). BAY 57-1293 inhibits the ATPase activity of the viral helicase-primase complex in a dose-dependent manner (IC_{50} of 30 nM).

The treatment potential of BAY57-1293 was investigated in several cutaneous and systemic animal models of disease (Betz *et al.*, 2002). The activities of orally administered BAY 57-1293 for the treatment of acute HSV-1 and HSV-2 infections were assessed in a widely-used murine lethal challenge model of disseminated herpes. Mice were infected intranasally and treated, starting 6 hours later for 5 consecutive days, three times a day. BAY 57-1293 and comparator drugs (ACV, GCV, VACV, FCV, Brivudin) were tested in escalating doses, and ED50 (dose at which 50% of the infected animals survive) values calculated. With an ED_{50} of 0.5 mg/kg of body weight TID against HSV-1 and HSV-2, BAY 57-1293 was the most potent compound tested. Comparable values for ACV were 22 and 16 mg/kg of body weight. No toxic side effects of BAY 57-1293 treatment were apparent in the mice upon gross inspection, and the highest dose tested (60 mg/kg TID) appeared to be well tolerated. Comparable results were shown in a rat lethal challenge model.

In the cutaneous zosterform model, oral treatment of HSV-2 established by dermal scarification was delayed until establishment of disease (day 3) and then animals were treated for 5 days TID with 15 and 60 mg of BAY 57-1293 per kg and 60 and 240 mg of VACV per kg. The lower dose of BAY 57-1293 was statistically more efficacious than the highest dose of VACV used ($P < 0.011$), based on a compiled disease severity score.

In the guinea pig vaginal model of HSV-2 genital herpes, delayed treatment with BAY 57-1293 (20 mg/kg 2 × daily orally; days 4–14 post infection) rapidly shut down disease progression. VACV at 7.5 × higher dose levels produced only a weak response. Benefit in terms of time to healing was clearly superior in the BAY 57-1293 treatment group. Perhaps the most promising results observed in the animal studies were the observation that acute treatment in this model could reduce the number of subsequent recurrences. This latter outcome may reflect the more potent and rapid shut down of the virus feeding the latency reservoir, and illustrates the importance of rapid diagnosis and treatment of the primary infection.

In these studies, the actual drug exposures (PK parameters) for BAY 57-1293 and comparators were not reported (Betz *et al.*, 2002) making it difficult to compare potencies. However, the PK for single 1 mg/kg dose in female BALB/C mice indicated high oral bioavailiability C_{max} 4.4 μM after 1 hour, and relatively slow elimination from the plasma ($T_{max} = 1$ hr; $t\frac{1}{2}$ 6 hours). Oral bioavailability >60% and an elimination half-life of >6 hours has also been observed in rats and dogs. Plasma concentrations with these properties would exceed 0.025 μM at 24 hours post dose. Under the conditions used in the cutaneous efficacy model studies with 15 and 60 mg doses administered 3 times daily, one would expect significant accumulations of drug levels above IC_{90} over the 5-day course of treatment; providing extended antiviral cover in the post-dosing period (Kleymann *et al.*, 2002). BAY 57-1293 shows the potential for once daily dosing, a convenience important for chronic suppression. From a pharmaceutical manufacturing perspective, such potency has advantages in smaller pill size and burden (number of pills per dose), resulting in economic savings in the amount of drug substance.

The long duration of drug exposure can offer advantages in terms of efficiency in reduced frequency of dosing, but must be balanced by an excellent safety profile

to avoid undesired consequences of toxic build up. Early toxicology studies indicated that once-daily dosing of dogs with 30, 100, and 300 mg/kg of BAY57-1293 for 28 days was well tolerated. The identical dosing protocol in rats, however, resulted in a dose-dependent transitional hyperplasia of the urinary bladder epithelium (Kleymann *et al.*, 2002). Based on a structural similarity to the diuretic drug, Diamox® (acetylzolamide), the Bayer toxicologists hypothesized that inhibition of the carbonic anhydrase enzymes led to bladder hyperplasia. Sulfonamides with broad inhibitory activity against the carbonic anhydrases of rats, dogs, and humans, only cause this bladder hyperplasia in the rodents. The in vitro inhibition of a carbonic anhydrase standard assay by BAY57-1293 occurs at 2 μM; 100-fold above the viral inhibitory concentration. Extended toxicologic evaluations will be required to further clarify this observation. The overall preclinical profile of this compound would support clinical development, and the evidence of superior potency and more rapid onset of action, and durability compared to the gold standard therapy, make it one of the most promising agents in the development pipeline (2004).

A novel series of inhibitors of the CMV helicase primase function was identified in a cell-based viral replication (single cycle) assay (Cushing *et al.,* 2006). The imidazolylpyrimidine core scaffold was substituted extensively at the 2-, 4-, 5-, and 6 positions to produce an active series of analogs with in vitro potencies ranging from 0.04–0.30 μM. The SAR revealed the importance of the imidazole/nitro group dyad, and the nature of the substituents at the 4 position. The chemical features were consistent with a mechanism of action involving a covalent binding to a target protein. Irreversible binding to the UL70 component of the CMV helicase primase complex (UL102, UL105, UL70) was demonstrated by co-immunoprecipitation of UL70 bound to radiolabeled inhibitor (Cushing *et al.*, 2006). Resistance selection is not yet reported for these new inhibitors. Two compounds provide excellent starting points for the further optimization for the necessary properties of a viable drug candidate.

Inhibitors of DNA processing and packaging

After herpesvirus DNA replication, the concatemeric product is packaged into preformed capsids and cut into unit-length genomes by site-specific cleavage. At least seven HSV proteins have been identified as participants in this process; pUL6, pUL15, pUL17, pUL25, pUL28, pUL32 and pUL33 (Beard *et al.*, 2002) and many of these have been confirmed as essential for viral replication. By analogy with DNA bacteriophage packaging and processing, a terminase complex binds to the capsid portal, trims the concatemeric DNA at a specific sequence with unique structural features (Adelman *et al.*, 2004), translocates the DNA into the capsid and finally cleaves the DNA at a repeat of the specific sequence. The HSV pUL6 protein has been shown to form the capsid portal (Newcomb *et al.*, 2001, 2003). A variety of evidence indicates that the pUL15 and pUL28 proteins form the terminase complex that cleaves the HSV DNA at the sequence before and after packaging (Beard *et al.*, 2002; White *et al.*, 2003; Przech *et al.*, 2003). The packaging genes are well-conserved among the herpesviruses; for example at least six of the seven HSV genes have close homologues in CMV. Of the terminase components CMV pUL89, pUL56 and pUL104 are the homologues of HSV pUL15, pUL28 and UL6 (Fig. 68.4). Biochemical and structural studies of the hCMV pUL89 and pUL56 suggest that the pUL56 binds to the viral DNA, while pUL89 mediates DNA cleavage via an ATP-dependent-nuclease activity (Bogner *et al.*, 1998; Scheffczik *et al.*, 2002; Scholg *et al.*, 2003). Since the processing and packaging of concatemeric DNA has no exact counterpart in the human cell this target presents the possibility of discovering very selective antiviral agents.

Inhibitors of the portal protein of HSV

WAY 150138

Activity of the thiourea class of compounds emerged from cell-based replication screens, revealing a striking degree of specificity within the alpha herpesviruses, but no activity across other human herpesviruses (Visalli and van Zeijl, 2003). Minor structural changes in the main scaffold resulted in >10-fold shifts in activity between HSV and VZV, and the lead HSV compound, WAY 150138, (benzamide, N-[3-chloro-4-[[[(5-chloro-2,4-dimethoxyphenyl)amino]thioxomethyl] amino]phenyl]-2-fluoro-). Identification of the portal protein as the molecular target was made by the generation and mapping of laboratory-derived resistant mutants (van Zeijl *et al.*, 2000; Visalli and van Zeijl, 2003). The portal proteins of HSV-1 and HSV-2 share 86% amino acid identity or similarity, while VZV (pUL54) and human CMV (pUL104) portal proteins share only 44% and 27% identity/similarity, respectively. Although these homologues share a high degree of functional homology, and strong overall amino acid identity in conserved domains, no broad spectrum inhibitors have yet been identified.

The individual mutations identified in the HSV-1 strains resistant to WAY-150138 suggested points of interaction with the compound resulted from the folding of the UL6 protein in its active 3- dimensional conformation. A

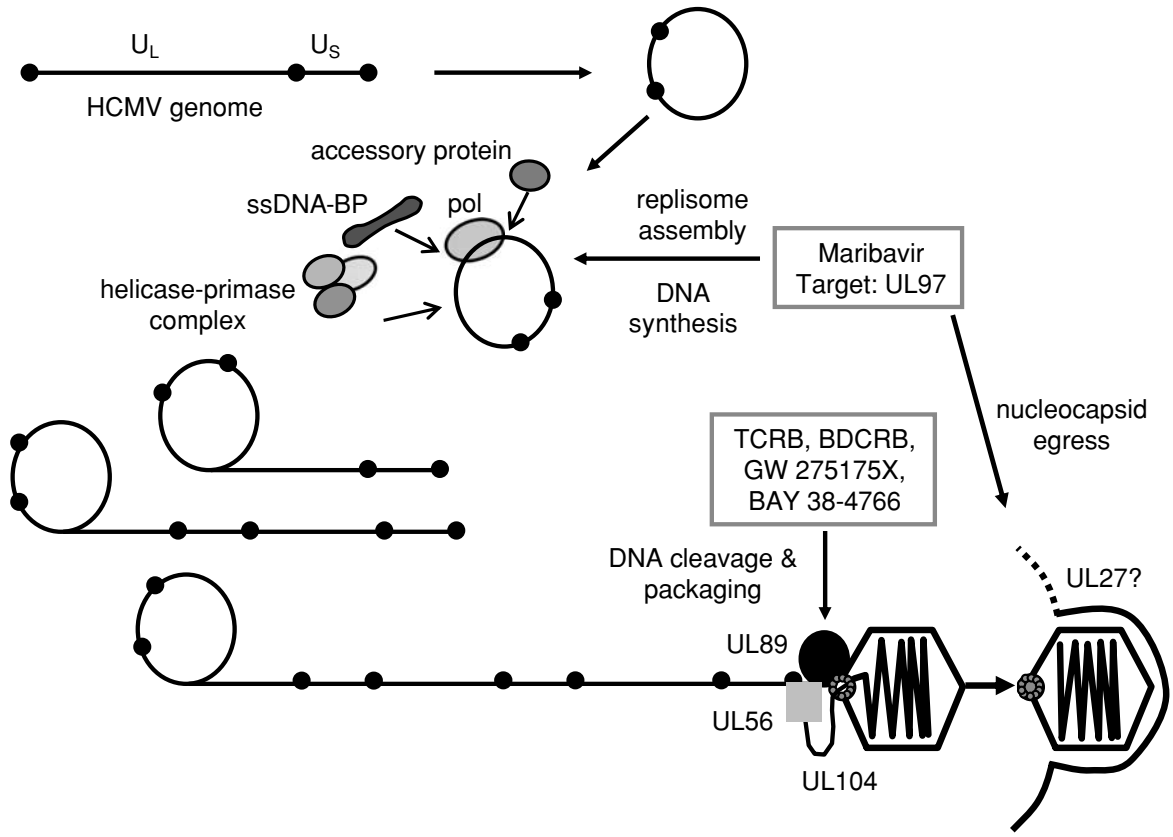


Fig. 68.4. Inhibitors of CMV DNA replisome, packaging, and nucleocapsid egress.

crystal structural model for the portal protein to aid in understanding the mechanism of UL6 binding and in further drug design was not available. Research on this class of portal protein inhibitors continues. No information is currently published on the other preclinical properties of these compounds that would indicate their potential as herpes simplex therapeutics.

Comp I

Subsequent screening of related compounds in the thiourea series revealed that a small chemical modification (addition of a spacer, -HC(CH3)-, between the aryl ring and the thiourea nitrogen) yielded compounds active against VZV, but with loss of activity against HSV. Three *N*-methylbenzyl-*N′*-arylthiourea analogues (Comp I, Comp II, and Comp III) were selected for further study.

A number of MOA studies were conducted confirming that VZV DNA cleavage and packaging is inhibited by these thiourea compounds via inhibition of the ORF54 gene (Visalli *et al.*, 2003). Resistant viral isolates were found to possess mutations in the VZV ORF54 gene, the homologue of HSV UL6, similar to the mechanism of WAY-150138 (van Zeijl *et al.*, 2000; Newcomb and Brown, 2002). As expected, treatment of wild-type virus with the inhibitor resulted in the absence of DNA-containing capsids, and restriction in the spread of infectious VZV to adjacent uninfected cells.

Current marketed VZV antivirals all target the DNA polymerase, and virus resistant to these new thiourea compounds are not cross-resistant to the approved drugs. Thus, targeted inhibition of the VZV ORF54 protein may prove to be a productive approach in identifying new agents to complement existing antivirals in the treatment of VZV infections.

Dihydroxyacridone series

The dihydroxyacridone series was investigated in order to target a MOA other than viral DNA polymerase (Akanitapichat *et al.*, 2000). Initial attempts focused on analogues with functional groups at the 5, 6, or 8 positions. The 5-Cl congener (5-chloro-1,3-dihydroxyacridone) was determined to be the most selective inhibitor of HSV and was selected for additional study. Mechanistic studies suggested that HSV replication was blocked after DNA and late protein synthesis. Further studies (Akanitapichat and

Bastow, 2002) indicated that maturation of replicating DNA and late virion production were inhibited in the same dose-dependent manner, resulting in a two- to three-fold reduction in the production of B capsids. Of interest was the inability to isolate resistant virus, although these attempts were limited, attempts to isolate resistant virus were unsuccessful.

Additional chemical elaboration and parallel synthesis of compounds in this series (Lowden and Bastow, 2003) identified compounds that were active against CMV (ED_{50} value of 1.4 μM at low MOI) and some that were active against both CMV and HSV. At least one compound in this series inhibited cell replication (mean CC_{50}s = 33 μM), but did not have antiviral activity. Preliminary mechanistic studies indicated the likelihood of diverse MOAs. While this has some appeal, more extensive work may be needed to determine the real selectivity and safety of this series of compounds.

Inhibitors of the CMV terminase complex

TCRB and BDCRB

The first selective inhibitors of the hCMV terminase complex, TCRB (2,5,6-trichloro-1-ß-D-ribofuranosyl benzimidazole) and its analogue BDCRB (1-(ß-D-ribofuranosyl)-2-bromo-5,6-dichlorobenzimidazole) arose serendipitously from a chemistry effort to modify the broad spectrum transcriptional inhibitor DRB into an anti-tumor agent (Townsend and Revenkar, 1970). The antiviral activity was uncovered during subsequent screening for antiviral activity in the series (Townsend *et al.*, 1995). In contrast to the action of GCV, viral DNA synthesis was unaffected in a single round of replication. The phenotype of infected, treated cells was consistent with a block in the viral DNA cleavage and packaging (Fig. 68.4).

Genetic mapping studies of the BDCRB and TCRB-resistant mutants of Towne and AD169 strains confirmed the interaction of these inhibitors with two subunits of the terminase complex (Krosky *et al.*, 1998; Underwood *et al.*, 1998). Specific point mutations were identified in the UL89 gene, which encodes the small subunit required for the nuclease cleavage of the precursor viral DNA into unit genome lengths (Bogner, 2002). A mutation was also selected in the UL56 gene, which encodes the large subunit responsible for sequence-specific DNA binding to the pac motifs, and ATP-dependent translocation of the viral DNA into the preformed capsids for final cleavage and packaging.

Precisely how two unrelated compound series, the β-D-ribosyl benzimidazoles and the sulfonamides, block DNA processing and packaging is not currently understood. The exact binding sites for BDCRB and BAY 38-4766 on the terminase subunits are apparently different, based on lack of cross-resistance (Evers *et al.*, 2004). Their binding could disrupt protein-protein interaction via allosteric mechanisms, or could directly interfere with enzymatic activity. The genome maturation process is complex, and occurs at the viral replication center sites, where viral and cellular transcriptional and DNA replication machinery also assemble (Dittmer *et al.*, 2005; McVoy and Nixon, 2005; Thoma *et al.*, 2006). BDCRB treatment of CMV-infected cells resulted in a major block to correct unit genome cleavage, and also allowed a minor level of monomer plus larger than unit product genomes resulting from skipped cleavages (McVoy and Nixon, 2005). This inhibitor also may directly interfere with the interaction of the pUL56 and the portal protein pUL104, as evidenced by the ability of BDCRB to block their co-immunoprecipitation from CMV-infected cells (Dittmer *et al.*, 2005). Consistent with a direct interaction of the pUL56 terminase subunit with the portal protein, resistance modifications in UL89 and UL56 to BDCRB, TCRB and BAY 38-4766 were accompanied by a compensatory change in UL104, which alone did not confer resistance (Buerger *et al.*, 2001; Reefschlaeger *et al.*, 2001; Komazin *et al.*, 2004). These interesting inhibitors continue to help elucidate the genome maturation events.

These two lead benzimidazole ribosides showed potent, selective activity for human CMV. However, pharmacokinetic studies indicated metabolic lability of the glycosidic bond between the base and the sugar moieties (Good *et al.*, 1994). Recently, the identification of two cellular enzymes that catalyze the cleavage of the glycosidic bonds of BDCRB and TCRB were reported (Lorenzi *et al.*, 2006). An active chemical program was undertaken to stabilize this linkage (Townsend *et al.*, 1999; Chulay *et al.*, 1999). The results of this effort were fruitful; yielding two clinical candidates, GW275175X, an inhibitor of the hCMV DNA terminase process packaging, and BW1263W94 (maribavir), an inhibitor of the pUL97 protein kinase (information as of 2003). The application of amino acid esters as pro drugs of BDCRB has also been successful, identifying L-Asp-BDCRB as a potential candidate for further development (Lorenzi *et al.*, 2005).

GW275175X

A benzimidazole with a pyranosyl sugar moiety selected for further development was **GW275175X** (2-bromo-5,6-dichloro-1-ß-D-ribopyranosyl-1-H-benzimidazole), a compound whose antiviral activity is specific for hCMV; there was no in vitro inhibition of HSV-types 1 or 2, VZV, or other DNA and RNA viruses tested (Williams *et al.*, 2003; Underwood *et al.*, 2004). MOA studies (Underwood *et al.*, 2004) supported inhibition of viral cleavage and packaging, consistent with the mechanism of BRCRB. As expected, virus with the BDCRB- and TCRB-resistant mutations in

UL89 were cross resistant to GW275175X. One notable aspect of the MOA is the rapid reversibility of the BDCRB or GW275175X block in infected cell culture, with resumption of viral DNA concatamer processing and restoration of the rate of viral yield production occurring within 8–10 hours following drug washout (Underwood *et al.*, 2004).

A battery of preclinical toxicology testing sufficient to support initial Phase I studies in humans (GSK, data on file) was completed for GW275175X. No significant adverse activity in in vivo assays designed to predict effects on metabolic parameters were exhibited. Other in vitro assessments of GW275175X were encouraging; pharmacology screens, and in vitro and in vivo mutagenecity tests were clean, and liver enzyme studies were clean. GW275175X was evaluated for toxicity in rodents (28-day acute, 6-month chronic) and primates (28-day acute). Systemic exposures at the various doses ranged from 60- to100-fold the in vitro IC_{50} for CMV inhibition. Importantly, penetration into the CNS and vitreous humor in primates was good, ranging from approximately 2 $\times$ the IC_{50} at 50 mg/kg/day to 8X the IC_{50} at 200 mg/kg per day. These characteristics would be important for treatment of congenital and ocular CMV disease.

Based on the overall preclinical properties and safety profile, GW275175X was progressed into Phase I single-escalating dose (100–1600 mg) studies. No drug-related or clinically significant changes from baseline were seen in vital signs, ECG, or clinical laboratory values, and all adverse events were considered mild. The pharmacokinetic profile indicated several advantages of this benzimidazole ribose over the parent BDCRB and the analogue, maribavir, including better CNS penetration, longer plasma half-life, and reduced serum protein binding.

While the overall preclinical and initial phase I data encouraged further clinical development, this compound was not advanced due to the sponsor's decision to progress the other anti-CMV agent from the benzimidazole riboside series (1263W94, maribavir) instead.

BAY 38–4766

The non-nucleosidic BAY 38–4766, 3-hydroxy-2,2-dimethyl-*N*-[4([[5-(dimethylamino)-1-naphthyl]sulfonyl] amino)-phenyl]propanamide compound emerged from a cell-based discovery program as the lead highly selective inhibitor of human, monkey and rodent CMV. Resistance to this drug was genetically mapped to the UL89 and the UL56 ORFs, indicating that this class of compound also targeted the hCMV terminase complex (Reefschlaeger *et al.*, 2001; Buerger *et al.*, 2001). The drug susceptibilities of 36 hCMV clinical isolates to the BAY 38–4766 and GCV were evaluated in two different phenotypic assays. All isolates including those resistant to GCV were inhibited by at least 50% at a concentration of approximately 1 μM of BAY-38–4776 (McSharry *et al.*, 2001a,b). Antiviral activity comparable to GCV was demonstrated in a hollow-fiber model where hCMV-infected human cells entrapped in hollow fibers were transplanted into immunodeficient mice (Weber *et al.*, 2001). Viruses resistant to BAY 38–4766 are not cross resistant to current marketed CMV drugs GCV, CDV or PFA.

A favorable pharmacokinetic profile has been demonstrated in humans for the initial lead compound in this series. A phase I study in healthy male subjects after single oral doses of BAY 38–4776 (100 to 2000 mg) indicated that the drug was well tolerated with no adverse events or changes in vital signs or lab parameters. The C_{max}s ranged from 0.33 mg/l (100 mg dose) to 4.2 mg/l (2000 mg/l dose) and occurred within 0.5 to 5 hours. After C_{max} concentrations were reached, drug was eliminated from plasma, with a $t_{1/2}$ of 3 to 5 hours reaching a terminal half life of 12–16 hours. The increases in AUC and C_{max} were dose dependent (Nagelschmitz *et al.*, 1999).

As of 2004, work continued to advance a compound from this interesting series into full clinical development.

Protease inhibitors

Impressive success in the development of protease inhibitors (PIs) in the treatment of HIV infection has not been paralleled in the herpesviruses. The PIs for HIV infection were introduced in 1996 and quickly set a new standard of care, dramatically extending the life of patients with HIV infection. Success in the design of therapeutic agents targeting the HIV protease is partly attributed to the well-defined structural features of the catalytic site in this aspartyl protease (Supuran *et al.*, 2003). In contrast, the herpes proteases belong to the serine protease family, and are characterized by a distinctive catalytic triad of his, his, ser within the less tractable active site. The cleavage sites are unique and highly conserved across the herpesviruses. The herpes proteases comprise the N-terminal sequence of the capsid scaffold protein. After completion of capsid assembly, auto cleavage by the protease releases the scaffold, permitting DNA packaging (Gibson *et al.*, 1994).

The herpes protease is essential for the production of infectious virus, and therefore represents a valid target. The quest for inhibitors of these proteases was facilitated by the development of efficient enzyme-based screens and wide-ranging X-ray crystallographic structure and catalytic site features that became available in the mid-1990s (Borthwick *et al.*, 1998; Holwerda, 1997; Qiu and Abdelmeguid, 1999; Waxman and Darke, 2000). Figure 68.5 illustrates the appli-

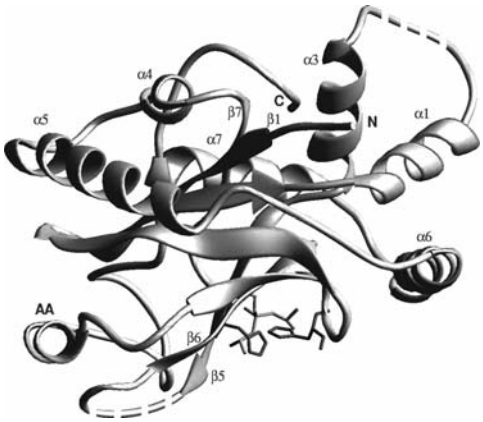


Fig. 68.5. The HSV-2 protease monomer. Two disordered surface loops are shown as dashed lines (N-terminal residues 1–16 are disordered). The two histidines in the active site are shown in red, one on the β6 and the other on the hairpin turn between β2 and β3, while the inhibitor diisopropyl phosphate covalently bound to the catalytic serine is in green on β5. (Reprinted with permission from Hoog *et al.*, 1997.)

cation of X-ray crystallographic structural information to the design and optimization of enzyme inhibitors (Hoog *et al.*, 1997), a tool used successfully in the HIV aspartyl PI discovery programs (Erickson *et al.*, 1990).

Several major pharmaceutical companies took up this quest, and several mechanism-based peptide and heterocyclic inhibitors of either the reversible or irreversible type were identified (Borthwick, 2005). However, these were not always active in virally-infected cells, and none have been progressed into clinical development to date. Any inhibitor would have to be capable of uptake not only into cells, but also into the capsid structure within the nucleus of the infected cells. This nucleocapsid barrier may contribute to the poor antiviral activity reported for compounds that are potent inhibitors of the enzyme assay in vitro (Borthwick, 2005). The protease as a target remains to be successfully exploited.

Inhibitors of the CMV UL97 encoded protein kinase

The CMV-encoded UL97 protein kinase has several features that make it highly attractive as a chemotherapeutic target. It belongs to a family of serine-threonine protein kinases highly conserved across all mammalian herpesviruses, suggesting its potential as a broad spectrum target (Chee *et al.*, 1989; Smith and Smith, 1989). The pUL97 shares structural features with aminoglycoside phosphotransferases; bacterial enzymes known to phosphorylate sugar-containing moieties, which may account for its fortuitous ability to monophosphorylate GCV and ACV (Littler *et al.*, 1992; Sullivan *et al.*, 1992; Talarico *et al.*, 1999; Zimmerman *et al.*, 1997; Michel and Mertens, 2004). This nucleoside phosphotransferase (ACV, GCV) activity has also been reported for the EBV homologue BGLF4 (Zacny *et al.*, 1999), but not for the alphaherpes virus kinase homologues. Importantly, the pUL97 differs from a prototypic serine-threonine protein kinase biochemically (high pH and NaCl optima) and substrate motif specificity (He *et al.*, 1997; Baek *et al.*, 2002).

The study of the replication functions of these herpes protein kinases is complicated by the fact that they are not absolutely essential for growth in cultured cells. Null mutants of the protein kinases of HSV-1, VZV, and CMV have shown variable phenotypes in different cell types, under different culture conditions, complicating MOA studies and predictably, quantitative drug inhibition studies (Moffat *et al.*, 1998; Chou *et al.*, 2006). Studies into the function of the HSV-1 pUL13 and the VZV gene 47 homologues indicate multiple activities throughout the virus replication cycle. These include regulatory roles in early gene expression, indirect effects on host gene expression, late protein post-translational modifications associated with virion maturation, and contributions to tissue-specific pathogenesis. (Purves *et al.*, 1993; Kato *et al.*, 2001; Kenyon *et al.*, 2001; Coulter *et al.*, 1993; Overton *et al.*, 1994; Ng *et al.*, 1994; Michel *et al.*, 1999; Kawaguchi *et al.*, 1999; Moffat *et al.*, 1998; Pritchard *et al.*, 1999; Wolf *et al.*, 2001; Krosky *et al.*, 2003b; Marschall *et al.*, 2005; Hu and Cohen, 2005).

Therefore, inhibition of such a target could cumulatively penalize viral replication. These proteins undoubtedly play essential roles in disease pathogenesis (Moffat *et al.*, 1998). In the case of the human CMV pUL97, the high degree of interstrain sequence conservation (Lurain *et al.*, 2001) coupled with the observations that there are no null mutants of the CMV pUL97 in clinical strains would argue that this is an ideal target. Two series of unrelated compounds are potent inhibitors of the CMV pUL97, and their MOA studies have extended our current knowledge of pUL97 function. Proof of concept for one of these has been achieved in the clinic.

Indolocarbazoles

Indolocarbazoles have been investigated as potential antiviral agents based on the fact that they are competitive inhibitors of the ATP binding sites of kinases in the protein kinase C family (Zimmerman *et al.*, 2000; Marschall *et al.*, 2001). A series of indolocarbazoles was recently analyzed and three (Go6976, K252a, K352c) were established to be highly effective inhibitors (IC_{50}s ranging from 0.009 to 0.4 μM) of both GCV-sensitive and -resistant hCMV,

with little effect against HSV. Cytotoxicity assays in proliferating cells reportedly indicated that the effective antiviral concentration of these compounds was significantly lower than those affecting cellular functioning. However, attempts to select resistant virus under the selective pressure of increasing concentrations of drug indicated that resistance was lost or restricted to low-level replication at higher drug concentrations, hinting that cellular functions may be involved (Kawaguchi and Kato, 2003). Alternative explanations include the possibility that resistance to Go6976 results in severe growth impairment, which could be established by the replication competence of a virus strain with deleted target. Growth impairment (1–2 logs titer reduction) has been reported for the deleted UL97 hCMV (Prichard *et al.*, 1999; Wolf *et al.*, 2001). Efforts to elucidate the exact MOA of the indolcarbazoles have focused on the hCMV pUL97 protein kinase. Indolcarbazoles with anti-CMV activity inhibited pUL97 protein kinase autophosphorylation in vitro, and mutant virus encoding a non-functional pUL97 (catalytic site mutant) deletion was completely insensitive to the indocarbazoles (Marschall *et al.*, 2001).

A series of symmetrical indolocarbazoles were independently synthesized to investigate SARs against a range of herpesviruses (Slater *et al.*, 1999). Several novel and potent inhibitors of hCMV were identified, although none were progressed to clinical development. Many of these had reasonable SIs in the normal diploid fibroblasts used for CMV activity, yet they were extremely toxic to human marrow stem cell differentiation. The development potential of this indolocarbazole class of compounds is unknown based on currently available information, which does not address pharmacokinetics and safety properties.

Recently a series of quinazolines with anti-CMV activity was shown to act through inhibition of the pUL97 (Herget *et al.*, 2004). Limited testing against cell protein Kinases indicated viral PK selectivity, although they also block host EGFR. The series appears promising, based on available data.

Maribavir

BW1263W94 1-(β-L-ribofuranosyl)-2-isopropylamino-5,6-dichlorobenzimidazole (maribavir), was derivatized from the original benzimidazole BDCRB and TCRB (Townsend *et al.*, 1995) as part of efforts to stabilize their metabolically labile glycosidic linkage and improve oral bioavailiability (Chulay *et al.*, 1999; Townsend *et al.*, 1995, 1999). The precedence of flipping the sugar conformation to the L or unnatural biologic form had been established in the nucleoside analog inhibitors of HIV RT as a way of reducing mechanism-based toxicity and rapid metabolic clearance. The potency of the β-L-BDCRB was amplified by various substitutions for the halogen in the 2-position of the benzimidazole base. The resulting development candidate BW1263W94 showed potent activity for CMV and EBV, but no other viruses, including the various animal CMV strains tested (Kern *et al.*, 2004). An unexpected finding was the change in the MOA: maribavir no longer exerted significant inhibition of viral DNA processing and packaging. Instead, maribavir strongly reduced viral DNA synthesis in the quiescent MRC-5 lung fibroblasts used in this study. The mechanism was not mediated through a substrate analog inhibition of the CMV DNA polymerase. Maribavir was not phosphorylated in infected cells, nor did the compound itself or any of its synthetic phosphorylated derivatives inhibit the CMV DNA polymerase (Biron *et al.*, 2002).

The discovery that maribavir was a selective inhibitor of the pUL97 came simultaneously from 2 independent approaches: selection and genetic mapping of a resistant virus strain, and fortuitously, from a broad protein kinase-inhibitor screening effort (Biron *et al.*, 2002). Resistant virus selected with a related analog in the series encoded a Leu397Arg amino acid substitution in pUL97, which conferred a 20- to 200-fold less sensitive phenotype to maribavir. This resistant virus remained susceptible to BDCRB and other approved anti-CMV drugs, including GCV, and was fully competent for in vitro growth. Supporting evidence for the pUL97 as the target was provided by studies with the pUL97 enzyme: the wild type pUL97-catalyzed phosphorylation of histone 2b was inhibited by maribavir (IC50 = 2 nM) while pUL97 with the Leu397Arg mutation was not (IC50 > 1000 nM). A second resistance mutation in the UL97 ORF was selected in the laboratory in the context of a clinical strain following serial passage in increasing concentrations of drug. The resulting point mutation encoded a Thr409Met change, which is also located close to the ATP-binding domain, and far upstream of the GCV resistance mutations which map to the substrate binding domain (Chou, 2006; Chou *et al.*, 2002). The resulting resistance phenotype was intermediate relative to that of the Leu397Arg mutation in AD169, conferring 80-fold vs. 200-fold increase (Biron *et al.*, 2002; Chou, 2006). Maribavir maintains activity against all GCV resistant UL97 mutants identified to date, indicating that the interactions of these two drugs with the protein kinase are distinct (McSharry *et al.*, 2001; Biron *et al.*, 2002).

Reduction in viral DNA synthesis by maribavir may be a consequence of inadequate or improper phosphorylation of pUL44 by the pUL97 kinase. The pUL97 carries a NLS, and locates to the nucleus during the replication cycle (Michel *et al.*, 1996). A direct interaction with, and phosphorylation by, pUL97 of the pUL44, the DNA processivity

factor, has been reported (Krosky *et al.*, 2003; Marshall *et al.*, 2003). This interaction was linked to the co-localization of pUL44 and pUL97 in the replication complexes (Marshall *et al.*, 2003).

The pUL97 has been shown to exert its major effects late in CMV replication (Wolf *et al.*, 2001; Krosky *et al.*, 2003a) based on studies with the RC∆97 (Prichard *et al.*, 1999). Consistent with studies of UL97 null virus, maribavir treatment of CMV-infected foreskin fibroblasts (HFF) also resulted in an increase of type A empty capsids (Wolf *et al.*, 2001) or type C DNA filled capsids (Krosky *et al.*, 2003a). These empty and precursor capsids accumulated in the nucleus of infected HFF cells at late times in the replication cycle, after infection with RC∆97, or in wild-type infected cells treated with maribavir. "Studies with HSV (Kato *et al.*, 2006; Simpson-Holley *et al.*, 2004) and with the CMV UL97 deficient virus (Wolf *et al.*, 2001; Krosky *et al.*, 2003a) have suggested a role for these viral protein kinases in nucleocapsid egress. The large nucleocapsids cannot exit through the nuclear membrane, which is composed of a tight structural matrix of proteins called lamins, without disassembly of lamin subunits in order to relax and open the junctions. Cellular p32 protein is reported to recruit pUL97 to the lamin B receptor, where it is hypothesized that pUL97 phosphorylates specific lamin components , resulting in their redistribution (Marschall *et al.*, 2005). Thus the viral protein kinase regulates a host protein substrate during virion maturation. The block of the CMV UL97 kinase activity by maribavir results in nuclear retention and accumulation of nucleocapsids (Wolf *et al.*, 2001; Krosky *et al.*, 2003a).

A second genotype has been associated with maribavir resistance following laboratory passage of both laboratory and clinical strains of CMV (Chou *et al.*, 2004; Komazin *et al.*, 2003; Chou, 2006). Mutations in the UL27 ORF, encoding changes of Arg233Ser, Ala269Thr, Leu335Phe, Leu335Pro, Trp362Arg, or Ala406Val-415stop, are reported to confer only a modest level of maribavir resistance (two-fold–5-fold increase in IC50s). Little is known about the function of pUL27 at this time. However, the UL27 homolog of mCMV, known as M27, was shown by mutagenesis studies to be non-essential for growth in culture, but was required for virulence and mortality in vivo (Abenes *et al.*, 2001). The CMV UL27 (1824 bp ORF, 608 amino acids) is transcribed as an early-late gene (Stamminger *et al.*, May 2002; Chou *et al.*, 2004), and has been shown to be nonessential for growth in vitro. The pUL27 encodes a nuclear localization signal (NLS); deletional mutagenesis resulted in cytoplasmic retention and a one-half log reduction in viral titers (Chou *et al.*, 2004).

Viral encapsidation and nuclear egress involve the action of a number of viral gene products (Mettenleiter, 2002), and the pUL97 clearly plays a role in the process. Data are accumulating which point to a role of the UL97 protein kinase action in CMV virion morphogenesis, perhaps by directing the normal intranuclear and intracytoplasmic distribution of viral proteins required for virion assembly and intracellular movement during the sequential steps of primary envelopment, tegumentation , translocation and final particle maturation (Azzeh *et al.*, 2006; Chou *et al.*, 2004; Prichard *et al.*, 2005). The pp65 antigen, product of the UL83 ORF, is phosphorylated by the pUL97 in transiently co-transfected cells, and its phosphorylation in cells infected with wild-type AD169 is blocked by maribavir , but pp65 phosphorylation is not blocked in cell infected with the maribavir-resistant strain Leu397Arg (Sethna, GSK data on file). Consistent with these findings, the intranuclear distribution of pp65 is altered in cells infected with the UL97 deficient strain, or in cells infected with wild type virus and treated with maribavir (Pritchard *et al.*, 2005). The pUL27 contains several proposed pUL97 substrate motifs (Baek *et al.*, 2002; Chou *et al.*, 2004). The elucidation of the role of pUL27 in the maribavir-resistant phenotype awaits further investigation on its function.

As maribavir progresses in clinical development, it will be important to understand the basis for drug resistance and its correlation with clinical outcome. The UL27 genetic changes that conferred resistance to maribavir resulted in only a low-grade resistance (three- to four-fold elevated IC50s) compared to the highly resistant phenotype of the L397RpUL97 strain. The gene sequence in 16 clinical isolates was 96% conserved, with no changes at the locations of the four UL27 maribavir resistance mutations noted. From a clinical perspective, the UL27 gene appears more tolerant of mutations than the UL97 gene (98% gene conservation; Lurain *et al.*, 2001), and the resulting levels of maribavir resistance may not preclude drug efficacy. It remains to be seen whether the prolonged drug selection pressure of prophylactic or suppressive regimens results in the accumulation of additional and multiple mutations in the UL27 and UL97 target genes, and potentially in as yet unidentified maribavir targets or UL97 substrates.

Preclinical toxicology testing of maribavir has been comprehensive, with overall results establishing a good safety profile (Koszalka *et al.*, 2002). These results contrast favorably with results of preclinical testing of GCV and CDV, both of which have a litany of toxicologic and tolerability concerns.

The pharmacokinetic profile of maribavir in rodents and primates demonstrated excellent oral bioavailability, although drug levels in the brain, cerebrospinal fluid, and vitreous humor of cynomologus monkeys were low. Maribavir is highly bound to human plasma proteins, principally

the albumin fraction; however, the binding is reversible in vitro. The impact of this property on efficacious dosing regimens remains to be determined.

Maribavir has successfully completed a series of Phase I–II clinical trials (Wang *et al.*, 2003; Lu and Thomas, 2004). In Phase I studies in healthy volunteers and HIV-infected subjects, single oral doses of 50 to 1600 mg produced similar dose-proportional pharmacokinetics. Maribavir was rapidly absorbed following oral administration with Cmaxs occurring within 1 to 3 hours. Absorption was at least 30% to 40%, and drug was eliminated from plasma with a t1/2 of 3 to 5 hours. The plasma and urinary excretion profiles indicated that the drug was extensively metabolized, with the major metabolite identified as the N-dealkylated analogue, which did not show anti-viral activity in vitro.

A pilot study was conducted in HIV patients ($n = 8$) with CMV retinitis, in order to measure the steady state pharmacokinetics in ocular tissues following 8 days of oral dosing. Antiviral drug levels were achieved in ocular tissues, although substantially lower than those found in plasma. This result indicates a potential for drug efficacy in CNS infections in congenital disease. As expected, blood CMV DNA levels in those viremic retinitis patients responded with a viral load drop. Additional Phase 1 studies included the important drug–drug interaction study using the drug cocktail approach to identify liver cytochrome P-450 enzymes capable of metabolizing maribavir, or being inhibited by maribavir (Ma *et al.*, 2006)

No safety concerns of note were observed in these studies; however, mild to moderate taste disturbance and headache were reported in 80% and 53% of the subjects, respectively (Wang *et al.*, 2003; Lu and Thomas, 2004). The taste disturbance was presumed to be due to secretion of drug or its principal metabolite metabolite into the salivary glands after systematic absorption and while not a safety concern, could have implications for adherence.

A subsequent phase I/II clinical trial was conducted to further determine the pharmacokinetics of maribavir and to monitor asymptomatic CMV shedding in semen in HIV-infected men (Lalezari *et al.*, 2002). Six dosage regimens (100, 200, or 400 mg three times a day, or 600, 900, or 1200 mg twice a day) or a placebo were evaluated for 28 days. In that proof of concept study, potent anti-CMV activity (2.9 to 3.7 $\log_{10}$ reductions in PFU/ml in semen) was established at all doses. The reductions in CMV titers for all regimens compared well with results reported for the approved doses of CDV (5 mg/kg) in a comparable trial (Lalezari *et al.*, 1995). Maribavir was reasonably well tolerated and safe with taste disturbance again the most frequently reported adverse event. Other adverse events reported by a higher percentage of subjects receiving maribavir than placebo included diarrhea, nausea, rash, pruritus, and fever.

Maribavir has recently demonstrated prophylactic efficacy in a Phase II randomized, double-blind, placebo-controlled trial in allogenic stem cell transplant patients (N = 111). Doses of 100 mg BID, 400 mg QD, and 400 mg BID for 12 weeks all reduced the rate of CMV reactivation; preemptive therapy was required for 15%, 30%, and 15% of the respective maribavir doses, relative to 57% for placebo recipients (ViroPharma Inc. press release March 29, 2006). The overall safety profile of maribavir in the 12 week study recapitulates earlier clinical results. The durability of the anti-viral effects and incidence of resistance is under study.

Maribavir is clearly the most advanced new anti herpes drug in the clinical development pipeline. Based on urgent medical need and the favorable development characteristics of maribavir, the FDA has granted Fast Track Status for the prevention of CMV infections in allogenic bone marrow and solid organ transplant patients (ViroPharma Inc. press release Feb. 7, 2005).

Antivirals with activity against EBV, HHV-6, HHV-7, and HHV-8

While anti-viral drug development efforts continue for HSV_1 VZV, and CMV utilizing both old and new targets, lack of progress against EBV virus is notable. There are several reasons for this. Primary EBV infection is generally subclinical in immunocompetent individuals. However, it may cause infectious mononucleosis (generally a benign and self-limiting disease) but the window of opportunity for antiviral treatment during the clinical course is short. The lytic EBV manifestation of oral hairy leukoplakia in immunocompromised patients responds to therapy with ACV. EBV-associated lymphoproliferative diseases, Burkitt's lymphoma and nasopharyngeal carcinoma, all of which may develop without obvious preceding immunodeficiency, remains a major unmet medical need. Latent EBV infection is considered either the etiologic agent for these conditions or a major contributing factor. (Thorley-Lawson and Gross, 2004).

From a drug development perspective; it is not yet clear what EBV or (host) functions to target; and the value of a viral replication inhibitor is unknown (Okano, 2003). The L-benzimidazole riboside, maribavir, which is in clinical developments for CMV disease, also shows activity against EBV in vitro, apparently by blocking both the appearance of linear forms of newly synthesized EBV DNA and the accumulation of the early antigen EA-D (Zacny *et al.*, 1999).

While the exact mechanism is unclear, it may be involve the EBV protein kinase, BGLF4, a close homolog of the hCMV UL97. This viral protein autophosphorylates, and has also been reported to phosphorylate the analogous DNA polymerase processivity factor, EA-D (Chen *et al.*, 2000). In lytically infected Akata cells, the level of hyperphosphorylated EA-D was reduced by maribavir treatment, similar to the impact of maribavir treatment on the hCMV pUL44. However, direct inhibition of the phosphorylation of EA-D has not been demonstrated, and maribavir did not block the phosphorylation of EA-D by EBV protein kinase in transient co-expression assays with these two viral genes (Gershburg and Pagano, 2002). Therefore the phenotype of maribavir-treated Alcata cells is consistent with inhibition of BGLF4 function; thus mechanism studies must be addressed within the context of the infected cell. While much remains to be determined regarding maribavir's mechanism of action against EBV, it should be considered a viable candidate for further study.

Information on the incidence of EBV-associated lymphomas in transplant patients treated prophylatically for CMV infections with maribavir may yield insights into the relationship of lytic replication to the post transplant lymphoproliferative diseases (Razonable *et al.*, 2005).

Research targeted at developing antivirals against human herpesvirus type 6 (HHV-6), type 7 (HHV-7) and type 8 (HHV-8) has been limited. A new series of arylsulfone derivatives have been shown to have in vitro activity against HHV-6 and HHV-7, as well as CMV. While work with this series of compounds is preliminary, a new MOA of indirect inhibition of viral DNA synthesis may be involved (Naesens *et al.*, 2006; Razonable *et al.*, 2005). Both HHV-6 and HHV-7 have an extremely high prevalence rate of about 95% in the US, but in the vast majority of cases their presence is associated with mild self-limiting symptoms, usually fever and rash, where treatment isn warranted. Infection with HHV-8 is more serious since reactivation is in the form of Kaposi's sarcoma (Ablashi, 2002). Immune preservation or reconstitution in AIDS patients by highly active anti-retroviral therapy (HAART) has reduced the incidence of Kaposis, indicting the protective effects of the functional immune system in this infection.

A systematic approach to identifying antivirals against HHV-6, HHV-7 and HHV-8 has been completed (de Clercq *et al.*, 2001b). Approved antivirals (ACV, VACV, PCV, FCV, GCV, PFA, CDV, and brivudin) and investigational compounds (Lobucavir, H2G, A-5021, D/L-cyclohexenyl G and S2242) with demonstrated activity against herpesviruses were evaluated in appropriate in vitro systems. The most potent compounds with the highest antiviral selectivity index were: (i) for HHV-6; PFA, S2242, A-5021 and CDV; (ii) for HHV-7; S2242, CDV and PFA; and (iii) for HHV-8; S2242, CDV and GCV.

Conclusions

The pipeline is indeed rich with new antiherpes agents. Many of these act by novel, and as yet unvalidated mechanisms of action from a therapeutic viewpoint. Their successful performance in the clinic will increase our understanding of the role of these new targets in viral disease.

For the two HSV helicase-primase inhibitors, a critical development milestone will be the human safety data for the thiazole class of compounds, the ease of viral escape (resistance), and the pathogenecity and transmissibility of mutant virus. The hCMV terminase inhibitor candidates start with the advantage of a highly selective and clearly essential target. However, it remains to be seen whether there are unanticipated (non-mechanism-based) toxicities in human studies with the two chemical series of inhibitors. Positive efficacy results in the Phase III clinical studies of the candidate CMV drug, maribavir, would provide target validation for the first inhibitor of a viral protein kinase.

As these drug candidates achieve regulatory approval, and with their expanded clinical use, the impact of drug potency and MOA on the emergence of resistance in the population will become the focus of studies. Resistance to the currently licensed antivirals (ACV, PCV, and their prodrugs) is low at <1.07 based on HSV isolates, a figure that has remained relatively constant since these drugs have been on the market, in spite of their widespread use (Bacon *et al.*, 2003). The vast majority of ACV-resistant HSV (TK-deficient) viruses are not capable of reactivating after latency, losing the transmission opportunity, and thus the presence of ACV-resistant virus in the overall population is acceptable. In an immunocompromised patient, the risk of developing resistance is much greater than in an immunocompetent individual, and clinical outcome becomes the critical issue. The availability of rescue therapies with novel MOAs will help fill this medical need.

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Part VII

Vaccines and immunotherapy

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69

Herpes simplex vaccines

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Introduction

Genital herpes, caused by both herpes simplex virus (HSV) types 1 and 2, can result in painful vesicular and ulcerative lesions on the genitalia and the genital tract, and may cause both urologic and neurologic problems. Following primary infection, HSV establishes a latent infection in the local ganglia and can reactivate on multiple occasions with manifestations ranging from asymptomatic viral shedding to painful recurrences of genital or orofacial lesions (Whitley, 2001). HSV-2 is the predominant etiologic agent of genital herpes and data from the second and third National Health and Nutrition Examination Surveys, spanning 1976 to 1994, demonstrated that the prevalence of HSV-2 infection increased by 30% since the late 1970's with the highest rates in teenagers and young adults (Fleming *et al.*, 1997). It is estimated that more than 1.6 million individuals are infected annually with HSV-2 in the USA (Armstrong *et al.*, 2001). From the perspective of vaccine development, however, genital herpes caused by HSV-1 cannot be overlooked. Although genital herpes caused by HSV-1 is generally less severe than HSV-2, HSV-1 is ubiquitous, infects a larger portion of the population than HSV-2, and the percentage of HSV-1 positive cultures isolated from individuals presenting with genital herpes appears to be increasing (Ribes *et al.*, 2001).

In addition to the pain and potential complications (e.g. psychological distress), that genital herpes causes in the infected individual, it is also responsible for increasing the risk of sexual transmission of HIV (Holmberg *et al.*, 1988). Shedding infectious virus during birth may infect the newborn and cause neonatal herpes (Wald *et al.*, 1995). Neonatal herpes caused by either HSV-1 or HSV-2, although relatively rare, can result in disseminated and/or central nervous system infections resulting in death or significant developmental impairments (Kimberlin *et al.*, 2001). The risk of neonatal herpes can be significantly reduced by Cesarean delivery when active lesions are detected immediately prior to birth; however in contrast to HIV transmission, subclinical shedding of infectious virus from the mother's genital tract during birth can cause neonatal herpes (Brown *et al.*, 2003). Despite the advances made in antiviral therapy over the past 20 years, genital herpes and neonatal herpes remain significant public health problems. Antiviral drugs can reduce the frequency of viral shedding and severity of symptoms associated with genital herpes and when given promptly can reduce some of the poor outcomes of neonatal herpes (Stanberry *et al.*, 1999; Kimberlin *et al.*, 2001). However, like many antiviral therapies, the effectiveness of the drug is limited by the development of viral resistance as well as delays in initiating treatment. Unfortunately, the awareness of neonatal herpes for acutely ill infants is low and the characteristic skin vesicles pathogenomonic for neonatal herpes do not occur in more than 30% of patients; both of these factors have contributed to the lack of progress in shortening the critical interval between diagnosis and treatment (Kimberlin *et al.*, 2001).

Cesarean delivery in high risk situations and antiviral treatment can reduce the total number of neonatal herpes cases; however, the frequency of inapparent genital lesions during delivery and the inability to quickly and specifically deliver antiviral therapy to many infected newborns combine to create an unacceptable burden of neonatal herpes cases (Stratton *et al.*, 2000). Moreover, infected women who do not have a history of genital lesions would not be targeted for antiviral prophylaxis, yet may excrete the virus. A vaccine approach that aims at reducing the overall burden of genital herpes and reducing the likelihood of viral transmission during birth should provide the largest public health benefit.

The feasibility of a vaccine approach is predicated on evidence that an immune response can positively modify the resulting disease. Specifically for neonatal herpes, maternal serostatus and the timing of infection relative to delivery has an impact on the frequency of neonatal herpes infections. Neonatal herpes occurred at a lower rate in neonates born to women who seroconverted prior to delivery than in neonates whose mothers did not seroconvert, demonstrating that the maternal immune status could impact the resulting disease (Brown *et al.*, 1997; Brown *et al.*, 2003). The mechanism of immune protection of the child remains unclear but transplacental transfer of maternal antibodies to HSV is likely to be important. In addition, serologic status also appears to have an impact on acquisition and/or outcome of infection with the heterologous virus type. Several studies have indicated that preexisting immunity to HSV-1, which is generally acquired early in life, can influence the outcome of HSV-2 infection at a later age (reviewed in Whitley, 2001). Individuals who were HSV-1 seropositive were not protected from acquiring HSV-2, but were approximately three times less likely to report a history of genital herpes than those who were HSV-1 seronegative (Langenberg *et al.*, 1999; Xu *et al.*, 2002). These data indicated that immunity elicited by prior infection with HSV-1 modified the disease due to the ensuing HSV-2 infection. Limited data suggests that the immune response elicited by HSV-2 infection may be even more robust than that elicited by a HSV-1 infection. Pregnant women with antibodies to HSV-2 were not infected with HSV-1 in contrast to women with no prior immunity (Stanberry *et al.*, 2002). Many of the vaccines in this review are derived from the antigens of HSV-2. These vaccines are expected to induce type specific immunity to the etiologic agent that is presumed to cause more significant disease as well as provide cross-protective immunity to HSV-1. Specific vaccines to prevent HSV-1 orofacial lesions have not been targets of significant development activity.

Notwithstanding widespread agreement that a vaccine will be the most cost effective way to reduce the morbidity associated with HSV infections (Arvin and Prober, 1997), no licensed vaccine is currently available to address the problems presented by these viruses. Development of herpesvirus vaccines present special hurdles associated with the complex replication cycles and propensity of these viruses to establish lifelong infections. Since much of the HSV disease burden is due to recurrent infection, a therapeutic as well as a prophylactic vaccine is needed. In addition, this family of viruses has also evolved mechanisms to counteract the immune response of the host. These cautions, however, are mitigated by the successful introduction of effective herpes vaccines such as Varivax® for the prevention of chicken pox and veterinary vaccines for Aujeszky's disease and Marek's disease, caused by alphaherpesviruses of pigs and chickens, respectively. This chapter will attempt to summarize the key steps and the more recent milestones in developing an effective vaccine for the prevention of disease caused by HSV-1 and -2.

History of HSV vaccine development

The history of HSV vaccine trials in humans is fast approaching the century mark with the ultimate goal of an efficacious vaccine still elusive. In the early to middle decades of the twentieth century, experimentalists used infectious wild-type viruses isolated from active lesions or passaged through animals to immunize individuals with the hope of having an impact on recurrent herpes. These approaches were later displaced by vaccine strategies using inactivated virus or glyocoprotein extracts. An excellent status report of previous vaccine development efforts that surveys the foundation of HSV vaccine strategies and that informs present day efforts is provided in a comprehensive review of a decade ago (Burke, 1993). For a variety of reasons including cost of clinical trials and difficulties in measuring clinical endpoints, many of these HSV vaccine candidates were not evaluated in rigorous placebo controlled, blinded, studies. Much more recently, Chiron Corporation and GlaxoSmithKline have sponsored double blind placebo controlled trials of two subunit vaccines that have put earlier basic and preclinical research vaccine concepts to the ultimate test. Other vaccine concepts need to be tested with similar rigor in clinical trials.

Development of live, attenuated vaccines

Live attenuated vaccines have many clear advantages as vaccine strategies. In principle, they can present the full range of all viral antigens to the immune system of the host, stimulating both the humoral and cell-mediated adaptive immune responses as well as innate immunity. This is an important consideration in rationalizing approaches to a vaccine because laboratory-based correlates of immune protection are not yet defined for the herpes simplex viruses. Generally, live vaccines evoke a longer-lived immune response than that elicited by other vaccine strategies. Additionally, powerful molecular tools exist for engineering recombinant vaccine viruses that can be employed to incorporate particular features designed to achieve the appropriate balance between attenuation and immunogenicity.

Developing a live, attenuated vaccine for HSV presents various challenges. HSV has evolved several mechanisms

to evade the host immune response, including functions that interfere with the production of interferon and products that inhibit the presentation of viral antigens to the host immune system (Johnson and Hill, 1998; Barcy and Corey, 2001; Lorenzo *et al.*, 2001; Leib, 2002; Hegde *et al.*, 2003). The advantage of removing these immune response modifiers has yet to be addressed in clinical trials. Removal could potentially increase the immunogenicity of the vaccine but it could also possibly create a scenario where superinfection or revaccination leads to significant reactogenicity. Another challenge to live vaccine approaches for HSV prevention is neurotropism. HSV establishes latent infections in sensory ganglia and can on rare occasion invade the central nervous system to cause encephalitis (Whitley, 2001). The optimal properties for a live, attenuated HSV vaccine candidate would remove the pathogenic signatures from the virus such that vaccination would not elicit any of the pain or ulcerative lesions typical of natural infection yet enable sufficient levels of replication in the host to elicit a vigorous immune response. In addition, the neurovirulence of the vaccine would need to be eliminated, although its propensity to establish a latent infection might be left intact. The ability of the vaccine to establish a latent infection could be viewed as a positive characteristic, since subclinical reactivation could restimulate the immune system, creating a more durable and effective immune response. However, delivering a vaccine that will establish a latent infection carries with it the concern that reactivation could result in transmission or disease at a time when the individual has become immunosuppressed due to infection or chemotherapy. In addition to these biological traits, any live HSV vaccine must be genetically stable and be able to be produced at sufficient quantities for effective administration.

Among the first live, attenuated HSV vaccines that were specifically manipulated to attempt to meet the above criteria were made by recombinant techniques. One of the viruses R7020 was constructed by replacing an approximately 14.5 kbp deletion extending from the α27 gene (UL54) in the unique long segment of the genome across the internal inverted repeats of HSV-1 with a fragment of HSV-2 encoding at least the gG glycoprotein (Meignier *et al.*, 1988). This construct was significantly attenuated in small animal models as well as in an exquisitely HSV sensitive nonhuman primate model (*Aotus trivirgatus*), and the neuroattenuation was genetically stable following serial passage through mouse brains (Meignier *et al.*, 1988, 1990). R7020 had a reduced propensity for establishing a latent infection and provided protection from direct challenge in animals. R7020 was evaluated in Phase 1 human trials. This construct was favored over a related *tk* negative construct (denoted R7017), since R7020 retained the *tk* gene and, therefore, retained sensitivity to available antiviral drugs such as acyclovir. Unfortunately, despite the promising immunogenicity results from the animal studies, the vaccine was poorly immunogenic in humans even given two doses of 10^5 PFU each and it was concluded that this vaccine was overly attenuated (Cadoz *et al.*, 1992). Although further development of these constructs as vaccines for immunocompetent individuals was aborted, they are still being evaluated as potential oncolytic agents for various cancer indications (for review see Varghese and Rabkin, 2002).

The next iteration of live attenuated HSV vaccines were developed based on the identification of a neurovirulence determinant identified as the $\gamma_1$34.5 gene. Removal of this gene, which is present in two copies in the genome of HSV, attenuated the neurovirulence of HSV-1 to a much greater extent than deletions of other specific genes that had been previously tested and did not significantly reduce the ability to replicate in cells that could be considered for vaccine production (Chou *et al.*, 1990; Whitley *et al.*, 1993). Experiments in small animal models demonstrated that this construct abolished the ability of the virus to migrate to and replicate in the central nervous system of mice and guinea pigs. An HSV-2 derivative of this construct denoted RAV 9395 was generated in which both copies of the $\gamma_1$34.5 gene were deleted as well as the ORFs antisense to the $\gamma_1$34.5 gene and the adjacent UL55 and UL56 ORFs. Since RAV 9395 was constructed from HSV-2, it was expected to elicit a more relevant immune response than the corresponding HSV-1 construct. RAV 9395 was tested in the guinea pig model of genital herpes and was shown to be attenuated and immunogenic. Following challenge of animals with the wild-type HSV-2 strain, animals previously vaccinated with 10^4–10^5 PFU of RAV 9395 were significantly protected from clinical disease compared to controls, and recurring lesions caused by the wild-type strain were reduced. Whether this was due to an inability to establish a latent infection in the dorsal root ganglia or a diminished ability to reactivate from this site is unclear (Spector *et al.*, 1998). The $\gamma_1$34.5 mutants were also characterized by their inability to grow in certain cell types *in vitro*, particularly those derived from nervous tissue. This characteristic, however, was not genetically stable in the HSV-1 background and could be reverted upon serial passage in these cells types (Mohr *et al.*, 2001). Further development of this or any recombinant based on deletion of the $\gamma_1$34.5 genes will require an analysis of the potential consequences of this instability, such as identifying compensatory mutations after serial passage and their effect(s) on attenuation. Recently, a vaccine candidate lacking both copies of γ134.5 as well as UL55, UL56 and the US10-12 region of HSV-2 (G) was shown to be genetically stable; this vaccine candidate remained highly attenuated following 9 serial passages in the CNS of mice. This highly

attenuated candidate protected guinea pigs from disease following challenge with the wild type virus (Prichard *et al.*, 2005).

Another set of approaches for making live, attenuated vaccines are based upon deletions or modifications to the viral genome that limit the replication of the virus except in specialized cell lines and have been denoted DISC, for disabled infectious single cycle mutants. In principle, a gene required for replication of HSV DNA or to produce infectious virus is removed from the genome and infectious virus is recovered on a cell line that provides the function *in trans*. Blocking different points in the replication cycle can alter the quantity and types of viral proteins expressed in a non-permissive infected cells (Farrell *et al.*, 1994; Da Costa *et al.*, 1997). Vaccine candidates with deletions of the gH, ICP8, ICP27 and ICP10 encoded functions have been constructed and tested. Recombinant HSV-2 viruses lacking the PK domain of ICP10 (the ribonucleotide reductase) have been tested in guinea pigs with favorable results. Exploration of the immune response in mice clearly demonstrated a Th1 type response characterized by development of both CD4+ Th1 and CD8+ CTLs with antiviral activity (Gyotoku *et al.*, 2002). Newer forms of DISC vaccines have been constructed in which two genes have been deleted and have been tested in mice. These modified vaccine elicited an immune response yet had significantly reduced quantities of latent viral DNA in the murine host (Da Costa *et al.*, 1999).

Preclinical evaluations of HSV-1 and -2 constructs lacking gH were tested in the guinea pig model and protected the animals in both prophylactic and therapeutic settings (McLean *et al.*, 1994; Boursnell *et al.*, 1997). A gH deleted HSV-2 strain HG52 was tested in a Phase 1 clinical trial to evaluate safety in HSV-2 seropositive and seronegative volunteers under the sponsorship of Cantab Pharmaceuticals. On the basis of acceptable safety and immunogenicity data reported for the prophylactic vaccine candidate, 485 patients were enrolled and vaccinated in a double-blind, placebo controlled Phase 2 trial of the DISC vaccine for the treatment of recurrent genital herpes. The results of this study have not yet been reported. The development of the prophylactic vaccine appears to have been discontinued.

Several of the DISC strategies are being exploited in the field of vectored vaccines. Nef antigens of simian immunodeficiency virus (SIV) were introduced into an HSV-1 mutant lacking ICP27. This replication incompetent vector elicited both cellular and humoral immune responses to HSV and SIV in rhesus monkeys and protected the animals following challenge 5 months post-vaccination (Murphy *et al.*, 2000). Many of the DISC viruses are being considered as potential vectors for use in gene therapy as well (reviewed in Rees *et al.*, 2002).

Subunit vaccines

Inactivated virus, glycoprotein extracts and recombinant subunit vaccines do not have several of the concerns associated with the development of live, attenuated vaccines such as neurotropism, transmission, or reactivation at a later time. Along with fewer safety concerns, a strength of these approaches is the ability to generate a well-defined biological product that can preserve conformational epitopes vital to eliciting an authentic immune response. A major consideration when choosing a subunit approach is the inclusion of an approvable adjuvant/antigen delivery system capable of eliciting both an antibody and a CTL response in humans.

Among the limitations of the subunit approach are the relatively narrow range of epitopes to which the immune system is exposed and the manner in which they elicit an immune response. Immunity generated to a specific target antigen may differ depending on how it is presented to the immune system and can also be affected by the HLA repertoire of the vaccinated individual. Strain variation coupled with HLA variability in an outbred population might influence the efficacy of the subunit protein vaccine in different subpopulations. The route of antigen presentation is another factor that must be considered, since an antigen introduced parenterally may elicit a different immune response than one that is expressed from a virally infected cell.

Many different inactivated HSV or glycoprotein preparations have been tested over the past several decades. Claims of positive benefit have resulted in use on a relatively large scale in some countries. Most of these claims, however, have not been substantiated by data from prospective, double-blind, placebo controlled clinical trials to establish safety and efficacy. Two recombinant subunit vaccine candidates, developed independently by Chiron and GlaxoSmithKline, are notable exceptions to this criticism. Both of these vaccines have been evaluated in relatively large, double-blind, placebo controlled trials to evaluate their impact on the occurrence of genital herpes disease or the prevention of new HSV-2 infection. The results of these studies will be described in more detail.

The Chiron subunit vaccine was made by expressing recombinant forms of two major surface glycoproteins of HSV-2, gB_2 and gD_2 lacking the carboxy-terminal regions, in Chinese hamster ovary (CHO) cells. The choice of

Table 69.1. Evaluation of recombinant subunit vaccine studies

	Dose (μg)	Regimen	Population	N	Vaccine effect reductions[a]	Comments
gB_2/gD_2/MF59						
	10	4 × 1 wk	guinea pig	15	Incidence, severity and mortality	Recurrence of challenge not reported
	30	0,1,6 mo	HSV-2^- Monogamous	531	No reduction in acquisition or severity	Transient reduction of acquisition in females only for the first 150 days of the trial
			HSV-2^- STD clinic	1862		
gD_2/ASO4						
	5	0,1,3 mo	guinea pig	15	Viral shedding, disease severity and recurrence	Incidence not reduced Mortality decreased
	20	0,1,6 mo	HSV-1^- / HSV-2^-	847	Genital herpes disease in HSV-1^-/HSV-2^- females only	Efficacy against genital herpes disease was not demonstrated in whole population or other subpopulations. Evidence of a trend toward prevention of infection in HSV-1^-/HSV2$^-$ females
			HSV-$1^{\pm}$ / HSV-2^-	1867		

[a] Observations with reported statistical significance.

these particular subunits relied on much basic research developed over the years that identified these glycoproteins as important stimulators of potent humoral and cellular responses (reviewed in Spear, 1985). These purified subunits were combined with the adjuvant MF59, a 5% squalene oil-in-water emulsion, to make the vaccine. The gD_2 subunit combined with MF59 was evaluated in the guinea pig model for its ability to protect the animals from a challenge with HSV-2. Animals that received 4 weekly doses of the vaccine developed significant serum IgG, as well as salivary, vaginal, and nasal IgA responses against HSV. Following intravaginal challenge with a lethal dose of HSV-2, vaccinated animals were significantly protected as measured by an decrease in disease incidence, severity and mortality (O'Hagan *et al.*, 1999).

A large clinical trial of the combination gB_2 and gD_2 subunit vaccine was conducted that evaluated the ability of this vaccine to prevent HSV-2 infection. The vaccine contained 30μg each of gB_2 and gD_2 combined with MF59 and given intramuscularly at 0, 1 and 6 months to adults who where seronegative for HSV-2 and were either attending an STD clinic or had a monogamous partner who was infected with HSV-2. The participants were followed for one year and acquisition of HSV-2 was measured by isolation of the virus in culture or seroconversion to HSV-2 proteins other than gB_2 or gD_2 (Corey *et al.*, 1999). Despite high titer HSV antibody responses, this vaccine had no impact on the overall frequency of acquisition of infection and did not modify the disease. The only difference between vaccine and placebo groups was a transient reduction in HSV acquisition in female subjects during the initial 150 days of the trial. The lack of impact on disease severity contrasted to an earlier study of this vaccine for a therapeutic indication. In the prior study, 212 adults with recurrent genital herpes were enrolled in a double-blind study and monitored for the number and frequency of recurrences as well as the duration and severity of each episode. As in the prophylactic trial, the number or frequency of recurrences was unchanged, however, the severity and duration of the first confirmed recurrence post vaccination was reduced significantly. (Straus *et al.*, 1997). The reason for these differences between the prophylactic and therapeutic trials is not clear but may relate to the difference between the natural history of a recurrent lesion that occurs when the host has immunity and a primary lesion in an individual who is immunologically naïve.

Another HSV subunit vaccine is under development by GlaxoSmithKline in which a purified carboxy-terminal truncated gD_2 expressed in CHO cells was formulated with the ASO4 adjuvant, containing aluminum hydroxide and 3-*O*-deacylated monophosphoryl lipid A (3-MPL). This vaccine was also evaluated in the guinea pig model. The vaccine was formulated by adding 5μg of gD2 to ASO4 and injected subcutaneously into the animals at 0, 1 and 3 months followed by an intravaginal challenge with HSV-2

two weeks later. Vaccination did not alter the incidence of infection, but it did reduce the titer of shed virus as well as the severity of the lesions and mortality. Recurrent lesions due to the challenge virus were also reduced when the number of episodes of recurrence was compared to controls (Bourne *et al.*, 2003).

This HSV vaccine was tested in a large double-blind randomized clinical trial to assess its ability to reduce the occurrence of genital herpes disease. Adults who were seronegative for HSV-2 were enrolled, vaccinated with 20 μg of gD_2 combined with alum and 3-MPL at 0, 1 and 3 months and monitored for the acquisition of genital herpes disease. The primary analyses did not show a vaccine effect. There was no reduction in the acquisition of genital herpes disease in HSV-2 seronegative adults who had received vaccine. However, post hoc analyses demonstrated that the vaccine significantly protected HSV-1 seronegative / HSV-2 seronegative women from acquiring genital herpes caused by HSV-2 (Stanberry *et al.*, 2002). In addition, although it did not reach statistical significance, there was a trend toward a reduction in HSV-2 infection in this same group.

Many of the differences between the results of the gD2/gB2/MF59 and gD2/ASO4 vaccine trials are enigmatic. Both vaccines protected guinea pigs from direct challenge with a high titered inoculum of HSV-2 indicating that these subunit compositions evoked similar immune responses in this animal model. Immunization of human subjects with gD2/gB2/MF59 resulted in the production of neutralizing antibodies as well as memory CD4+ T cells specific for the antigens in the vaccine (Langenberg *et al.*, 1995). However recent studies suggest that this vaccine may have induced only low levels of antibodies that mediate antibody-dependent cellular cytotoxicity in humans (Kohl *et al.*, 2000). The gD2/ASO4 vaccine also elicited high levels of neutralizing antibodies in humans as well as gD specific lymphoproliferation and interferon γ (Stanberry *et al.*, 2002). These adaptive immune responses, however, were not different between the groups protected from acquisition of genital herpes and those that were not. The reason for the efficacy of this vaccine in women and not men as well as the lack of effect in females who were HSV-1 seropositive will require additional studies; there is no obvious explanation for these differences. The positive results in HSV-1 seronegative/HSV-2 seronegative women in this trial have prompted GlaxoSmithKline Biologicals in conjunction with the National Institute of Allergy and Infectious Diseases to initiate a pivotal Phase 3 efficacy trial in 7,550 women who are seronegative to both HSV 1 and HSV 2.

Other approaches to vaccination

Many options for the development of prophylactic HSV vaccines are currently being explored in preclinical studies and several have progressed to immunogenicity studies in the guinea pig model of HSV. DNA vaccines that expressed either a full length or secreted, carboxy-terminal truncated form of gD2 reduced the lesion severity and number of recurrent lesions following challenge (Strasser *et al.*, 2000). Other more novel approaches such as ISCOMS (immunostimulating complex particles) made from detergent extracted preparations of virions have been tested in guinea pigs (Simms *et al.*, 2000), and attenuated Salmonella vaccines harboring a gD plasmid have been evaluated in rodents and shown to protect the animals following challenge (Flo *et al.*, 2001). Peptide vaccines are being evaluated based on the identification of immunologically dominant epitopes, but to date most have not developed past studies in the mouse model (Gierynska *et al.*, 2002; BenMohamed *et al.*, 2003). Approaches using chimeric DNA vaccines combining viral glycoprotein epitopes, protein fusions of viral glycoproteins with bacterial open reading frames, or with chemokines are being tested in mice. Alternative adjuvants such as CpG molecules are being added to formulations of peptide and DNA vaccines. Vectored approaches using other viruses offer other vaccine strategies. For example, VZV has been used as a vector to express HSV gD, and has been shown to protect against experimental HSV challenge in a guinea pig model (Heineman *et al.*, 1995). Further development of these new approaches as well as new ideas should continue to be advanced with time.

Conclusions

The goal of a safe, efficacious HSV vaccine has several components. Clearly the most easily measured benefit is to reduce the rate of acquistition of genital herpes disease. Reduction of the incidence of this disease or reduction of viral shedding from infected individuals should have a direct impact on the number of neonatal herpes cases every year as well as on the sexual transmission of HIV. The two most recent large clinical trials of subunit vaccine candidates have highlighted some of the issues that may need to be addressed during the development of a successful HSV vaccine. Gender differences influenced the vaccine efficacy as did the preexisting HSV-1 serological status. The mechanisms of these interactions are not understood at this point and the influence of these differences on other vaccine

strategies cannot be predicted. Further studies to understand how these factors affect vaccine efficacy should be done. In addition, genital herpes caused by HSV-1 appears to be increasing and HSV-1 can also cause severe neonatal herpes. A successful vaccine should confer immunity to both etiologic agents. Ultimately, the role of gender, preexisting immunity, and effectiveness for preventing both HSV-1 and HSV-2 disease and the impact on public health will be needed to evaluate HSV vaccine candidates.

Animal studies and serological assays as measures of the immune response may not be sufficient to completely predict the functional behavior and immunogenicity of a vaccine in humans. The immunogenicity of R7020, was poor in humans and the gD2/gB2/MF59 vaccine, while stimulating robust neutralizing antibodies was unable to protect individuals from acquisition of HSV-2 despite the promising immunogenicity and protection in mice and guinea pigs. These results highlight the need to further explore the mechanisms of functional immunity in humans at a fundamental and detailed level in order to define informative surrogate markers for the clinical development of an HSV vaccine candidate. In addition, the efficacy trials of the subunit vaccines confirmed that prevention of infection is a difficult endpoint to achieve and may misdirect the effort away from relieving the burden of disease associated with genital herpes. The potential for transmission of herpes during birth as a consequence of asymptomatic viral shedding despite vaccine induced immunity will have to be considered to predict the impact of candidate vaccines on neonatal herpes.

Although a specific vaccine and immunization strategy have not yet been identified for HSV, the overall progress made by the field has brought an increased understanding of the complexity of HSV-1 and HSV-2 disease and elucidated many issues that can now be addressed. A vaccine for human papilloma virus (HPV), another sexually transmitted disease, has recently been shown to have positive results in Phase 2 clinical trials (Koutsky *et al.*, 2002; Galloway, 2003). These virus-like particle vaccines have provided significant immunity to prevent establishment of a persistent infection. Adding an HSV vaccine to an armamentarium of STD vaccines would provide a major positive impact on public health throughout the world.

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Varicella-zoster vaccine

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Varicella vaccines: background

A live attenuated varicella vaccine, the Oka strain, was developed by Takahashi and his colleagues in Japan the early 1970s (Takahashi *et al.*, 1974). This vaccine is now being adminstered to varicella-susceptible healthy children and adults in many countries; it is produced by at least 3 manufacturers worldwide (Merck and Co., Glaxo SmithKline, and Biken Institute/Aventis Pasteur). Although the vaccine was developed in Japan, the largest experience with it comes from the United States, where the Merck formulation was licensed for routine use in healthy susceptible individuals over the age of 1 year in 1995 (Centers for Disease Control, 1996). In both pre- and postlicensure studies (Gershon *et al.*, 1984a, b; White, 1997; Sharrar *et al.*, 2000) the vaccine was demonstrated to be extremely safe. Adverse effects in healthy persons are few and quite transient: a sore arm after the injection in 20%–25%, and a very minor rash resembling mild varicella in about 5%, usually appearing a month after immunization (White, 1997). A small proportion of vaccinees (15%) may also experience mild fever. It takes about a week to demonstrate antibodies to varicella-zoster virus (VZV) after immunization, but protection often results even after an exposure has occurred. As a result of widespread immunization of children, the epidemiology of varicella has begun to change in the United States, with a reported marked decline in incidence in sentinel areas, where active surveillance for the disease is being carried out (Seward *et al.*, 2002). Vaccination is now being explored for the possibility of its preventing or modifying zoster. In a very real sense, the development of live varicella vaccine paved the way for the development of other vaccines against herpesviruses.

A number of possible misconceptions concerning varicella and varicella vaccine are listed in Table 70.1. This manuscript will discuss these misconceptions in the framework of the history of development of the live vaccine and its record regarding safety and efficacy of prevention, with regard to varicella as well as zoster.

History of development of the live attenuated vaccine

In 1974, when the first publication concerning the Oka varicella vaccine appeared, there was considerable controversy concerning whether use of a vaccine against a herpesvirus was likely to be safe and could possibly be effective. In Japan, the strategy used was first to vaccinate healthy children and then to gradually try to immunize safely immunocompromised children, progressing from the mildly to the severely immunocompromised. This possibly protective approach was taken because at that time, many young children were surviving cancer only to die of varicella. In studies in Japan, involving less than 200 healthy and immunocompromised children, it was found that the Oka strain appeared to be safe, although more needed to be learned about its efficacy. Because immunocompromised children in the Untied States were similarly faced with the possibility of surviving malignancy but not surviving chickenpox, interest in the Oka strain began to increase during the decade of the 1970s. While vaccine use remained controversial in the United States, most experts in the field believed that the risk-benefit ratio had become, by the late 1970s, appropriate to explore the safety and efficacy of varicella vaccine in children with underlying acute lymphoblastic leukemia in remission, because they had a 7% mortality from varicella (Feldman *et al.*, 1975). Studies showed that the vaccine could be used safely in these high-risk children, although some developed fairly extensive vaccine-associated rashes that required treatment with antiviral drugs (Gershon, 1995). Importantly, the vaccine

Table 70.1. Misconceptions concerning varicella vaccine and its use

Varicella and zoster are invariably mild diseases.
The vaccine is not very efficacious.
Zoster will become epidemic with widespread vaccination.
Varicella will cause more disease in older age groups if vaccine is given routinely to children.
The vaccine is too expensive to use.
The vaccine is not safe.

proved to be highly protective against varicella, despite the weakened immune systems of these children. Because of the high-risk nature of varicella in children with malignancies, it was not possible to conduct a controlled efficacy study. Vaccine efficacy, however, could be tested because of the high clinical attack rate of varicella in susceptible children following a household exposure to VZV(Gershon *et al.*, 1984a,b). As data regarding the clinical efficacy of the vaccine began to emerge, significant interest developed in determining if the vaccine would prove safe and effective in healthy children, thus eventually eliminating much of the necessity to immunize immunocompromised children.

Virology of the attenuated Oka strain of VZV

The original virus was isolated from a 3-year-old, otherwise healthy Japanese boy with varicella. To prepare the seed lot, the virus was passaged 11 times at 34 °C in human embryonic fibroblasts, 12 times at 37 °C in guinea pig fibroblasts, and 5 times in human diploid fibroblasts (WI-38 and MRC-5 cells) at 37 °C. (Takahashi *et al.*, 1974) Additional passages were carried out by the manufacturers to prepare the vaccine to be marketed. Because VZV is so strongly cell-associated, the final product had to be sonicated and clarified by centrifugation to produce live cell-free virus.

Fortunately, it was possible soon after the vaccine was developed, to be able to distinguish between the wild type virus and the Oka strain, which made possible the reliable analysis of clinical information following vaccination. Initially it was necessary to propagate VZV from a rash or other body tissue or fluid in order to identify whether the vaccine type virus was implicated when a complication of vaccination was suspected (Gelb, *et al.*, 1987). Eventually, however, it became possible to distinguish between vaccine and wild type VZV using polymerase chain reaction (PCR) without having to resort to virus isolation. (LaRussa *et al.*, 1992; Gomi *et al.*, 2000, 2001; Loparev *et al.*, 2000a, b) A number of mutations have been found to be present in the Oka vaccine strain that are not present in the parental Oka virus (Gomi *et al.*, 2000, 2001; Loparev, *et al.*, 2000a, b). Although it is known that most of the mutations in the vaccine strain are in open reading frame (ORF) 62, exactly which mutations are associated with attenuation have not yet been identified. The Oka strain replicates less efficiently in human skin than does wild type VZV, as studied in the SCID-hu mouse model (Moffat *et al.*, 1998).

Based on the vast quantity of clinical experience with the live VZV vaccine, there is overwhelming clinical evidence that the vaccine virus is attenuated. Both the incidence and severity of rash following vaccination compared to natural infection are decreased by a factor of about 20, whether vaccination is given by injection or inhalation (Bogger-Goren *et al.*, 1982; Gershon, 2001). Transmission of the Oka strain from healthy vaccinees with rash to other susceptibles is extremely rare and has been reported only on 4 occasions (LaRussa *et al.*, 1997; Salzman *et al.*, 1997; Sharrar *et al.*, 2000). This is in marked contrast to the extraordinarily high degree of contagion of the wild type virus in which roughly 90% of susceptibles develop clinical varicella after family exposures (Ross *et al.*, 1962). Finally contact cases of Oka varicella, which occur with some frequency after vaccinating leukemic children as well as rarely from healthy populations, are extremely mild or subclinical (Tsolia *et al.*, 1990; Sharrar *et al.*, 2000). Transmission has only occurred when the vaccinee has manifested a rash due to the Oka strain, and there has been no clinical evidence of reversion of the vaccine strain to virulence.

Safety of the varicella vaccine for healthy individuals

With any preventive medical intervention that is to be used widely on a routine basis, particularly in children, safety is the major concern. Live varicella vaccine has proven to be extremely safe when given to susceptible children, as indicated by extensive pre- and postmarketing studies in the United States. Prior to licensure over 9000 healthy children and 2000 adults were safely immunized in clinical trials (Gershon, 1995; White, 1997). Adverse effects were minor and transient.

A post-marketing safety study of the vaccine was begun at its licensure in 1995, by investigators at Merck and Company and at Columbia University (Sharrar *et al.*, 2000). Medical providers and consumers were asked on a voluntary basis, to submit information on possible adverse reactions

to the vaccine they observed. In addition to clinical information, samples of rashes and other possibly involved fluids and tissues were submitted to analyze first for VZV by PCR, and then to distinguish between the two types of virus if VZV was identified (LaRussa *et al.*, 1992). Although these data were collected passively, and the information therefore is necessarily incomplete, important data emerged from the study. It was crucial to be able to distinguish between vaccine and wild-type viruses because the temporal relationship of vaccination and development of rash and other symptoms would seem to implicate the vaccine virus without laboratory identification. Had it not been possible to distinguish between the two viruses, many adverse events would have erroneously been attributed to the vaccine type virus.

In the first 4 years after licensure, over 16 million doses of varicella vaccine were distributed in the United States. In the postmarketing study covering this period, rash was the most frequently reported adverse event, and almost all rashes consisting of more than 50 skin lesions were found to be caused by the wild type virus. There were fewer than five patients with more than 200 skin lesions shown to be caused by the Oka strain; this is below the average number seen in children experiencing natural varicella. There were 19 reports of encephalitis and 24 reports of ataxia during the year following vaccination, but the Oka strain of virus was not implicated in any of these illnesses. Wild-type VZV was, however, implicated in one patient with ataxia and one with encephalitis. Although there have been many reported fatalities due to wild type VZV, there have been no reports of fatal VZV infection caused by the Oka strain (Sharrar *et al.*, 2000).

A handful of serious disseminated infections have been reported in children who were thought to be immunologically normal when vaccinated, but were eventually identified as immunodeficient. The varicella vaccine virus remains sensitive to acyclovir and other antiviral drugs. All of these children received antiviral therapy and recovered from the VZV infection. These include one child each with human immunodeficiency virus (HIV) infection and essentially no CD4 lymphocytes (Kramer *et al.*, 2001), asthma and high dose steroids (Sharrar *et al.*, 2000), adenosine deaminase (ADA) deficiency with immunodeficiency (Ghaffar *et al.*, 2000), neuroblastoma diagnosed and treated right after vaccination (Levin, 2003), and a deficiency in natural killer cells (Levy *et al.*, 2003).

Zoster has been reported after vaccination but infrequently. In the first 4 years after licensure, the Oka strain was identified in 22 patients and wild type VZV in 10 with zoster (Sharrar *et al.*, 2000). To date, after distribution of over 40 million doses in the United States, there have been less than 50 reported cases of zoster shown to be due to the Oka strain (Galea *et al.*, 2002).

Immunogenicity of varicella vaccine in healthy children and adolescents

In prelicensure studies, children under 12 years of age had a seroconversion rate of 97% after one dose of vaccine, as determined by the exquisitely sensitive glycoprotein immunosorbent assay (ELISA) 6 weeks after immunization. This test is not available on a commercial basis. Adolescents and adults had a seroconversion rate of 82% after one dose of vaccine, which rose to 99% after 2 doses (Provost *et al.*, 1991; Gershon, 1995). A seroconversion rate of 91.5% was noted after one dose of vaccine, using an immune adherence antibody assay (IAHA), in 2565 Japanese children immunized between 1987–1993 (Asano, 1996). Unfortunately, commercially available ELISAs are neither sensitive nor specific enough to detect reliably antibodies after VZV immunization (Saiman, *et al.*, 2001). There were a number of reports of failure to seroconvert (using a commercial ELISA test) in healthy children and adults in the Merck-Columbia post-licensure study (Sharrar *et al.*, 2000). Most of these probably represent a failure of the test used rather than of the vaccine itself. The most sensitive and reliable method to measure antibodies to VZV, the fluorescent antibody to membrane antigen test (FAMA), is not amenable to performance on a large scale, and therefore remains a research tool (Williams *et al.*, 1974; Gershon, 1995). In studies in leukemic children, this assay indicated a seroconversion rate of 82% after one dose of vaccine and 95% after two doses (Gershon *et al.*, 1996). A convenient test that is sensitive and specific to reliably measure VZV antibody titers on a large scale basis continues to be sorely needed.

Efficacy and post-licensure effectiveness of varicella vaccine

Early studies in vaccinated leukemic children who were in remission from their illness and usually received two doses of varicella vaccine indicated not only that the vaccine was safe but also that it was highly protective against varicella (Gershon *et al.*, 1984a,b). About 85% of vaccinated leukemic children were completely protected against varicella after household exposure and those who developed breakthrough infection had mild disease requiring no antiviral therapy. In contrast, one would expect that, in varicella-susceptibles, about 90% would become obviously

infected following a household exposure to the virus (Ross *et al.*, 1962). Early studies in healthy children indicated a similar degree of protection against household exposure, following 1 dose of vaccine, with breakthrough disease in about 15% (White, 1996).

Two double blind placebo-controlled studies of varicella vaccine in healthy children were performed, involving a total of about 1500 children. Both indicated that the vaccine provided protection of about 90% (Weibel *et al.*, 1984; 1985; Varis and Vesikari, 1996). In these studies, higher doses of vaccine (10 000–17 000 plaque forming units-pfu) were associated with better protection than lower doses (1000 pfu). Many different doses of varicella vaccine have been studied in various clinical trials. The currently licensed Merck vaccine contains about 3000 pfu per dose, and the Glaxo SmithKline (GSK) vaccine contains about 10 000 pfu at the time of release, which prior to expiration date falls to about 3000 pfu. The Merck vaccine is lyophilized and frozen, while the GSK product is lyophilized and stored in the refrigerator (Gershon *et al.*, 2002). No direct comparison of efficacy of these vaccines has been performed, and therefore it is assumed that they are similar in efficacy. Clearly both are highly efficacious.

A postlicensure case-control effectiveness study involving PCR-proven cases of varicella in otherwise healthy children examined the performance of varicella vaccine in clinical practice in New Haven, CT. This study indicated that the vaccine in the US is about 85% effective in preventing all varicella, and virtually 100% protective against severe varicella in otherwise healthy children (Vazquez *et al.*, 2001). There were 202 children with varicella and 389 matched controls. Of these, 23% with varicella and 61% of controls had been vaccinated (vaccine effectiveness 85%). Of 56 vaccinated children with varicella, 86% had only mild disease; in contrast 48% of the 187 unvaccinated children had mild varicella. Studying the vaccine as it is used in clinical practice is especially significant because the vaccine itself is labile and loses potency if it is not stored properly, as indicated in the package insert, lyophilized and frozen.

Perhaps the best indication of the effectiveness of varicella vaccine, however, is the reported dramatic decline in the disease since 1995. This has been observed in sentinel areas of the USA (Seward *et al.*, 2002). Varicella is often mild and uncomplicated in otherwise healthy children, but it may unpredictably be associated with significant morbidity and even mortality. In the United States, in the prevaccine era, there were about 100 annual deaths from varicella and and 11 000 hospitalizations. Most reported deaths from varicella occurred in otherwise healthy individuals. Beginning in 1995, active surveillance of varicella in three sentinel counties in Texas, California, and Pennsylvania, was carried out by investigators at the Centers for Disease Control (CDC). Vaccination coverage in the year 2000 in the sentinel counties in children aged 19 to 35 years of age ranged from 73.6% to 83.8%. For the period of study, the number of cases of varicella and hospitalizations decreased sharply, with a reduction ranging between 71%–84% in the 3 counties. Hospitalizations for varicella per 100 000 persons decreased from 2.7–4.2 in 1995–1998 to 0.6 in 1999 and 1.5 in 2000. The long-standing recognized seasonality of varicella with increases in incidence of disease in winter and early spring also disappeared after 2000. The decrease in varicella occurred in individuals of all age groups including infants too young to be immunized and also adults, who were less likely to be immunized than children, indicating that herd immunity had developed (Seward *et al.*, 2002) (see figure in Seward chapter).

A prospective study of the incidence of varicella in 11 day-care centers in North Carolina between 1995–1999, indicated a similar decrease in the incidence of varicella due to vaccination. A case control analysis of this population indicated vaccine effectiveness of 83% (Clements *et al.*, 1999). In a further analysis of this population, the rate of varicella vaccine coverage increased from 4.4% in 1995 to 63.1% in 1999. The incidence of varicella per 1000 person–months fell from 16.74 in July 1996 to 1.53 cases in December 1999. Because the decrease in varicella disease exceeded the increase in vaccination rate over the period studied, the investigators proposed that herd immunity had occurred (Clements, *et al.*, 2001).

Considerations of vaccine use

In the United States, contraindications to varicella vaccine include pregnancy, allergy to vaccine components, and immunodeficiency. It is recommended that children receiving doses of steroids of over 2 mg/kg per day of prednisone or its equivalent NOT be immunized unless this medication can be discontinued for at least 3 months before vaccination. There are currently no programs for immunization of children with underlying leukemia because of potential safety concerns. On the other hand, studies of children with infection with human immunodeficiency virus (HIV) have indicated that it is safe to immunize them as long as their CD4 lymphocytes exceed 25% of their total lymphocytes in their peripheral blood. Two doses of vaccine are given to HIV-infected children, 4–8 weeks apart (Levin *et al.*, 2001). The CDC has supported this recommendation (Centers for Disease Control 1999). Children undergoing renal transplantation have been safely immunized in French studies. These children have had protection

against varicella and also have had a decreased incidence of zoster, compared to similar children who experienced natural varicella (Broyer and Boudailliez, 1985a, b; Broyer *et al.*, 1997).

Because transmission of the vaccine virus to others is rare, healthy persons who have close contact with susceptible individuals who are at high risk to develop severe varicella are recommended to be immunized. This includes, for example, healthy children whose pregnant mothers are susceptible to varicella, and children whose varicella-susceptible siblings have malignant diseases for which they are being treated.

While the major thrust of vaccine use in the United States is in young children who have not been exposed to the virus, the vaccine often provides protection to susceptibles who have already been exposed. That post-exposure vaccination can be successful was best demonstrated in studies in Japan in the 1970s and 1980s (Asano *et al.*, 1977, 1982). In these studies, family members who were exposed to varicella were immunized within 3 days and the disease was largely prevented. Vaccination was also used successfully to control an outbreak of varicella in a shelter for homeless families that was experiencing an epidemic of chickenpox (Watson *et al.*, 2000).

The vaccine has been shown to be cost effective in a number of studies in the United States and abroad (Lieu *et al.*, 1994; Beutels *et al.*, 1996; Burnham *et al.*, 1998; Coudeville *et al.*, 1999; Diez Domingo *et al.*, 1999; Brisson and Edmunds, 2002). In general this is a vaccine that is geared for use in developed countries, where it is not uncommon for children to be cared for out of the home as both parents are employed, or in single parent households where there is only one breadwinner.

Persistent questions regarding varicella vaccine

While ideally a vaccine should induce protection of close to 100% against a given disease, breakthrough cases of chickenpox have consistently been reported despite the administration of varicella vaccine, an observation first made in the early clinical trials involving leukemic children (Gershon *et al.*, 1984a, b). The rate at which breakthrough varicella has occurred has varied from study to study. There are a myriad of possible explanations for the phenomenon. One obvious one is that not even natural varicella induces total immunity in every individual. Second cases of natural varicella are well recognized to occur (Gershon *et al.*, 1984a, b; Junker *et al.*, 1989, 1991). It is unrealistic to expect a viral vaccine to provide better protection than the natural illness itself. Another indication that complete immunity to VZV may never be quite achievable is the existence of zoster, which is due to reactivation of latent VZV in persons with partial immunity. Unless a vaccine that does not induce latent infection is developed and widely used, herd immunity will be required to control diseases due to VZV in addition to personal immunity from the vaccine itself.

There is now general agreement that varicella vaccine is both safe and effective. A number of important questions about the vaccine have been raised recently. None of these uncertainties preclude the use of varicella vaccine, but all need further exploration. The remainder of this chapter will address these issues.

Does immunity to varicella wane with time after immunization?

There are two types of vaccine failure, which have been termed primary and secondary. Primary vaccine failure, commonly called a "no take," is said to occur when there is no measurable immune response to a vaccine that was administered and the person remains susceptible to the immunizing product. Persons who received varicella vaccine and developed full blown infections which are severe have probably experienced this phenomenon. In the Merck-Columbia postmarketing study, there were 11 reports of severe varicella despite immunization (Sharrar *et al.*, 2000). How often primary vaccine failure occurs is unknown, but it should be recalled that even with the very sensitive gp ELISA antibody test, the seroconversion rate in healthy children was 97%, not 100%. Considering that about 4 million children are immunized annually in the United States, there would be expected to be over 100 000 children annually who might have primary vaccine failure. This might be true for other vaccines as well and underscores the need for strong herd immunity in protection against infections after vaccination.

Secondary vaccine failure is said to occur when an immune response brought about by vaccination decreases with time, leaving the vaccinee with varying degrees of susceptibility to the disease. At present, there is little evidence for secondary vaccine failure due to waning of immunity to VZV after immunization of healthy children, but subtle degrees of waning immunity may be difficult to identify. An extremely high degree of persistence of antibodies and cellular immunity to VZV have been reported for as long as 20 years after vaccination, in Japanese and American studies (Asano *et al.*, 1994; Arvin and Gershon, 1996; Ampofo *et al.*, 2002). Moreover, studies in over 400 vaccinated adults indicate that there is no increase in the incidence or severity of breakthrough varicella with time, with up to 20 years of

follow-up (Ampofo *et al.*, 2002). Were immunity to be waning, one would expect that breakthrough disease would become more frequent and more severe with time after vaccination, but this has not been observed.

Nevertheless there are hints that the current vaccine strategy in the U.S. may need some adjustment to provide better protection, especially to young children. It is disquieting that about 10% of children may develop a modified form of varicella despite vaccination, and in some studies the rate has been even higher. In four studies, the breakthrough rate of varicella in children followed for up to 10 years after immunization, ranged between 18%–34% (Clements *et al.*, 1995, 1999; Johnson *et al.*, 1997; Takayama *et al.*, 1997). This breakthrough varicella may occur months to years after immunization, and it is caused by wild type VZV (LaRussa *et al.*, 2000). It may be so mild that it is misdiagnosed clinically as insect bites or hives. It occurs mainly in individuals who have low VZV antibody levels following immunization.

A number of outbreaks of varicella in vaccinated young children have been reported in the United States (Dworkin *et al.*, 2002; Galil *et al.*, 2002a, b; Gershon, 2002). Some of these cases may have resulted from secondary vaccine failure. In addition, there are other possibilities to explain these outbreaks. The vaccine is labile, and improper storage may account for primary vaccine failure in some children. Children with asthma may have less ability to mount a protective immune response, possibly related to medications such as steroids (Izurieta *et al.*, 1997; Shapiro and LaRussa, 1997). Currently, a CDC study to examine the seroconversion rate to VZV in vaccinated asthmatic children, as determined by FAMA, is underway in the United States, which should provide an answer to this question. In two reports, children vaccinated when they were less than 15 months old had higher rates of breakthrough varicella than those immunized when they were older (Dworkin *et al.*, 2002; Galil *et al.*, 2002a, b). When varicella vaccine is administered with an interval of less than one month after another live vaccine has been given, the incidence of breakthrough varicella increases (Centers for Disease Control, 2001) (Table 70.2).

One report of an outbreak of chickenpox in a day-care center in New Hampshire, in which the rate of vaccination in attendees was high is especially compelling with regard to the possibility of secondary vaccine failure (Galil *et al.*, 2002a, b). In this outbreak, the effectiveness of the vaccine was only 44%, much lower than in any previous report. In this study, 25/88 (28.4%) of children developed varicella in a 6-week period. It seems likely that the index case, whose varicella was quite extensive, had experienced primary vaccine failure. In the other involved children, the only factor that was associated with vaccine failure was an interval of greater than 3 years since vaccination. However, the children involved were very young, and it may be that the age at vaccination combined with the interval of time after immunization also played a role in predisposing them to breakthrough varicella. Continued investigations will be necessary to further understand whether waning immunity is a significant factor in breakthrough disease and how frequently it might occur, but it seems to be a real possibility. A follow up of the study conducted in private practices in New Haven has indicated that vaccine efficacy decreases with time, from 97% in year 1 to 72% in year 6 (Vazquez *et al.*, 2003), which confirms and extends the above observations of Galil *et al.* (2002a, b).

Table 70.2. Factors associated with vaccine failure/breakthrough disease

Improper storage of vaccine
<14 months old when immunized
Asthma
<30 days between MMR and varicella vaccine
>30 months since immunization
Low vaccine dosage

Table 70.3. Factors associated with successful vaccination

High vaccine dosage (over 10 000 pfu)
Two doses of vaccine, which provide higher antibody titers which in turn correlate with better protection

It may be that a second dose of varicella vaccine given routinely will alleviate potential problems of primary and secondary vaccine failure (Gershon, 2002). (Table 70.3) One possible way in which this could be accomplished in a practical manner is to administer routinely two doses of vaccine as measles, mumps, rubella, varicella (MMRV). Developing an immunogenic formulation of the varicella component of MMRV has proven to be a difficult task. Even a formulation of very high titered VZV (40 000 pfu) did not produce significantly higher titers of VZV antibodies, although it appeared to be safe (Shinefield, *et al.*, 2002). However, MMR and varicella vaccine may be administered safely together, and many children receive the two vaccine formulations at the same time although in separate syringes at different body sites (White, 1996).

It should be emphasized that breakthrough varicella is almost always a mild infection. Reports of varicella of normal severity in vaccines are few and probably represent mostly episodes of primary vaccine failure (Sharrar *et al.*,

Table 70.4. A comparison of the reported percentage of immunocompromised patients developing zoster who were vaccinated and those who experienced natural infection

Underlying diagnosis	Interval of observation in years	Vaccinees N (%)	Natural varicella N (%)	Reference
Leukemia	6	(6)	(19)	Takahashi *et al.*, 1990
Leukemia	6	34 (0)	73 (21)	Brunell *et al.*, 1986
Leukemia	10	96 (4)	96 (16)	Hardy *et al.* 1991
Renal transplantation	10	212 (7)	415 (13)	Broyer *et al.*, 1997

2000). Zoster may be more of a concern although it appears that zoster is more common after natural than vaccine infection (see below). If skin lesions of varicella predispose to latency, as has been suggested by experimental evidence (Chen *et al.*, 2003), then it would be important to try to prevent breakthrough infection in order to minimize the chance of developing zoster.

Varicella-susceptible adults may be safely immunized against varicella. Two doses are utilized, 4–8 weeks apart. Adults manifest lower seroconversion rates after one dose of vaccine than children, and therefore two doses are routinely administered. As in children, adverse effects are minimal and protection is high (Gershon, *et al.*, 1988; 1990; Ampofo *et al.*, 2002).

Serologic testing following immunization is usually discouraged because VZV antibody tests are both insensitive and results may be non-specific. Whether adults with no history of varicella should have serologic testing prior to immunization or simply be given two doses of vaccine if they have no history of past varicella remains moot. Inadvertent administration of vaccine to persons with immunity to varicella is not harmful (Gershon *et al.*, 2002).

Zoster: effects and potential effects on its incidence in the vaccine era

Although use of varicella vaccine is now contraindicated in immunocompromised individuals, early vaccine studies often involved these children because they were at high risk of developing severe varicella. (Table 70.4) In these vaccinees, the incidence of zoster was shown to be lower than after natural infection (Hardy *et al.*, 1991; Arvin and Gershon, 1996). Thus, there is every reason to predict that vaccination would also be protective against zoster in healthy children. Although the data are of necessity not controlled, the existing information since 1995 to the present strongly suggests that this is the case. Although healthy vaccinees have developed zoster, less than 50 known cases have been reported after distribution of over 40 million doses of vaccine between 1995 and 2002 (White, 1996; Sharrar *et al.*, 2000; Galea *et al.*, 2002). The rate of zoster after vaccination is roughly 20 times less the expected rate for that age group (Gershon *et al.*, 2002).

Recently several studies have addressed the question of whether exposure to varicella is protective against zoster, because it is known that zoster is associated with a low cell-mediated immune response to VZV. It was found that in vaccinated leukemic children, both household exposure and additional doses of varicella vaccine correlated with greater protection against zoster than a single dose of vaccine alone (Gershon *et al.*, 1996a, b). A case-control study has shown that following natural varicella, there is a lower incidence of zoster in individuals who have exposures to children with VZV infections in comparison to those who do not (Thomas *et al.*, 2002). Based on such data, using computer modeling, a theoretical calculation of the incidence of zoster in a highly vaccinated population has been made. This model predicts an epidemic of zoster with accompanying significant mortality in countries where varicella vaccination is routine (Brisson *et al.*, 2002). These observations, even though theoretical, have led to reluctance to use varicella vaccine routinely in some countries. It is therefore important to put the possibility into perspective.

The reported incidence of zoster in healthy individuals aged 40–50 in developed countries ranges between 2–4 cases per 1000 person–years of observation (Hope-Simpson, 1965; Donahue *et al.*, 1995). It was calculated that the rate of zoster would double in countries where routine vaccination is being carried out (Brisson *et al.*, 2002). This would lead to an incidence of 4–8 cases per 1000 person–years of observation in this age group. The incidence of zoster per 1000 person–years of observation in other high risk groups has been reported to be as follows: vaccinated leukemics 8, unvaccinated leukemics 25, adults with AIDS 50, children with AIDS 300 (Gershon *et al.*, 1996a, b). Thus the projected increase in the incidence of zoster based on computer modeling might double but even this is unlikely to represent an epidemic. Moreover, the mortality of zoster appears to be less than that from

varicella, the primary infection, and thus the validity of an increasing incidence of zoster leading to significantly increased mortality must be questioned (Feldman *et al.*, 1973; 1975; Whitley *et al.* 1982a,b; Shepp *et al.*, 1988). Finally, as yet, no actual increase in the incidence of zoster has been observed in the United States although the CDC is collecting epidemiologic data on the issue. Should an increase in zoster be recognized, however, it can logically be approached by immunization to prevent zoster, as described below.

Vaccination to prevent zoster in the elderly

It is estimated that about 20% of individuals who have had natural varicella will develop zoster during their lifetime, usually if they become immunocompromised or elderly. After age 50, the incidence of zoster climbs steadily with each advancing decade. While mortality from zoster is rare, morbidity from this infection remains a significant medical problem. The risk of developing the severe and painful complication of post-herpetic neuralgia (PHN) also increases with increasing age. It has been recognized for years that zoster occurs when the cell-mediated immune (CMI) response falls to below a critical level, and with advancing age, fewer and fewer people maintain a positive CMI response to VZV, although their antibody titers remain intact or may even increase (Arvin *et al.*, 1978; Berger *et al.*, 1981; Gershon and Steinberg, 1981; Burke *et al.*, 1982; Hardy *et al.*, 1991).

In attempts to explore whether vaccination may be used to boost immunity to VZV and possibly be used to prevent zoster, at least 8 clinical trials have been performed by investigators in the United States and Europe. These trials have determined that it is possible to boost CMI responses safely in many, although not all, elderly subjects. Varying doses of vaccine from roughly 1000 to 12 000 pfu have been employed. Some of these studies employed controls and others did not; in each, the subjects and medical personnel was aware of whom had received which vaccine. While no firm conclusions can be made from these studies, it appeared that vaccination seemed to modify zoster. All observed cases of zoster were mild in these otherwise healthy adults (Levin, 2001).

Based on these apparently successful open label studies, a large double blind placebo controlled study of immunization of healthy individuals over the age of 60 is currently being carried out. This study has now enrolled approximately 30 000 subjects and the observational period is still in progress. After a 3-year follow-up interval, the code will be broken and the data will be analyzed. At present, no results from this study are available, but there should be some published information by 2005. The vaccine employed is the live attenuated product, at a dose of about 20 000 pfu, which is roughly ten times the dose that is administered to healthy children (Levin, 2001) (see update below).

Use of inactivated varicella vaccine in patients at high risk to develop zoster

As in the studies described above to try to prevent zoster in elderly individuals, these studies were undertaken to try to boost the CMI response to VZV in highly immunocompromised patients who are at even greater risk to develop zoster than elderly patients. Because of the possibility of inducing another serious VZV infection with the live vaccine itself, however, an inactivated formulation of the vaccine was utilized. In an early controlled study, three doses of heat-inactivated vaccine were administered to a heterogeneous group of 75 patients; the incidence of zoster remained unchanged but the illness appeared to have been modified (Redman *et al.*, 1997). In a second, more successful clinical trial, 4 doses were employed, including one given a month before transplantation, and the subject population was more homogeneous and included only patients with lymphoma who had undergone autologous stem cell transplantation (Hata *et al.*, 2002). A dose of 6115 pfu of heat-inactivated VZV was administered, which was well tolerated although induration, erythema, or pain occurred after 10% of the doses. The rate of zoster was significantly decreased in vaccines compared to controls. Zoster occurred in 17/56 (30%) of the evaluable unvaccinated control patients and in only 7/53 (13%) vaccinees. Protection correlated with reconstitution of CD4 T-cell immunity to VZV. Because all the patients who developed zoster received antiviral therapy for their illness, it was not possible to compare the severity of zoster in the vaccines and controls. While this vaccine remains experimental, it holds promise for eventual prevention of zoster in immunocompromised patients. It also suggests an approach that might successfully prevent infections with other herpesviruses in the immunocompromised (Hata *et al.*, 2002).

Recent developments

There are two recent changes in policy with regard to recommendations for VZV vaccine use in the United States. They involve a CDC mandated second dose of varicella vaccine for all children, and the use of the newly licensed

by the FDA of combined measles-mumps-rubella-varicella (MMRV) vaccine. An additional development concerns vaccination of healthy older adults against zoster. Recently it was demonstrated that zoster can be prevented by immunization of healthy individuals over the age of 60 years with a different formulation of the Oka vaccine VZV strain that contains over 10 times the dose of virus as the monovalent varicella vaccine.

As has been mentioned, by 2002, numerous outbreaks of varicella were being observed in immunized children in day care facilities and schools in the United States. Vaccine efficacy rates were calculated to vary from 85% to as low as 44%.(Galil *et al.*, 2002b) One study showed that it appeared that the vaccine failure rate after 1 dose of vaccine might be as high as 20%, which alone could account for apparent breakthrough disease. In 2006, a study of the seroconversion against VZV of 16 month old children vaccinated in a practice setting at Vanderbilt University in 2004, indicated a seroconversion rate of 76% (FAMA $\geq$ 1:4) 16 weeks after 1 dose of Varivax (Michalek, Gershon *et al.*, personal communication). As part of a study conducted by Merck study (Kuter *et al.*, 2004), roughly 1000 children received a second dose of varicella vaccine 3 months after the first dose. A similar number of children were followed for the same interval but received only 1 dose of vaccine. In these children who received 2 doses, the seroconversion rate 6 weeks after the second dose increased to 99.5%, and the geometric mean titer (GMT) to 141.5, indicating a marked booster response after the second dose. In this study, the gp ELISA assay was used to assess humoral immunity. In October, 2006, the FDA approved the use of the combined vaccine MMRV for use in the United States. Marked boosting of humoral immunity following a second dose of MMRV was also observed (Shinefield *et al.*, 2005a,b). Boosting against VZV was about 10 times greater than boosting against the MMR components, in which it was only about 2 times greater. Importantly, as part of the Merck study (Kuter *et al.*, 2004), protection against varicella over 10 years of follow up was significantly higher after 2 doses of vaccine than 1 dose, 94% vs 98% respectively.

Due to the costs, inconvenience, and transmission of wild-type VZV associated with the numerous outbreaks of varicella, as well as the consistently observed boosting of both humoral and cell mediated immunity after a second dose of vaccine, the ACIP mandated a second routine dose of varicella vaccine for infants and children, with catch up programs, in June 2006. For harmonization with MMR vaccine, MMRV will usually be given at 12–15 months of age with a second dose at 4–6 years of age. Details concerning this recommendation should become available on the CDC website in the near future. As yet this information is unpublished.

Without a second dose of vaccine it was predicted that there would be an accumulation of vaccinated children destined to become varicella-susceptible young adults, potentially at high risk to develop severe varicella. This would especially be liable to occur in the setting of less opportunity for boosting of immunity due to less exposure to cases of varicella. In addition, it had been found by the CDC that outbreak control with a second dose was expensive and almost impossible to implement. While it is uncertain that there will be complete protection against varicella after 2 routine doses of varicella vaccine, it is projected that the numbers of breakthrough cases will decrease significantly, with a subsequent decrease in transmission of the virus, and a decrease in outbreaks.

Monovalent varicella vaccine contains a dose of about 1350 plaque forming units (pfu) of virus, but MMRV contains about ten times this amount. The increased dose of varicella vaccine in MMRV was required in order to reach an acceptable seroconversion against VZV. It is hypothesized that the measles component in MMRV may suppress the immune response to VZV. This dose of virus in MMRV is similar to the dose of vaccine used to prevent zoster.

In 2005, it was shown in a double-blind placebo-controlled study that vaccination of healthy adults over age 60 years resulted in significant protection against zoster and that in preventing zoster, postherpetic neuralgia was also prevented (Oxman *et al.*, 2005). In vaccinees aged 60–69 years, the vaccine was 64% effective in protection against zoster. Although the vaccine was less effective in preventing zoster in vaccinees aged 70–79 years (41%), it was 55% protective against PHN in this age group. Many questions about the use of this vaccine remain, such as the duration of immunity, but thus far the vaccine appears to be safe and effective when used in this manner. Currently the decisions are being made as to exactly how this vaccine should be used in the elderly population; the outcome should be known in the fall of 2006 (see CDC website for further information).

Conclusions

Despite the controversial introduction, varicella vaccines represent the first truly successful preventive measure against VZV. Although the approach of routine vaccine regimens for children may require some adjustment, the vaccine safely prevents most cases of clinical varicella, which saves lives, hospitalizations, and resources. Recipients of live vaccine are also at decreased risk to develop zoster. The

promise of prevention of zoster in individuals who already have latent infection with the wild type virus due to past natural infection with VZV is a potential goal that is likely to be accomplished with either live or inactivated vaccine or both. A new era in herpesvirus virology is well underway.

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Human cytomegalovirus vaccines

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Efforts to develop a human cytomegalovirus (HCMV) vaccine began more than 30 years ago in response to then recent reports that HCMV was capable of causing severe congenital disease. During the intervening years, our understanding of HCMV biology and immunology has increased dramatically. That knowledge, coupled with the introduction of several new vaccine methodologies, opened the door to an impressive expansion of HCMV vaccine research, particularly during the past decade. This chapter focuses on the principles underlying HCMV vaccine development and on the vaccine approaches that are currently under investigation.

Cytomegalovirus and human disease

The manifestations of HCMV infection vary with the age and immunocompetence of the host. In both adults and children, HCMV infection is usually asymptomatic. On rare occasions, otherwise healthy adults with primary HCMV infection will experience an infectious mononucleosis-like syndrome, with prolonged fever and mild hepatitis (Cohen and Corey, 1985). However, HCMV can cause serious morbidity and mortality when the host is unable to mount an adequate immune response or when infection is acquired in utero.

Congenital HCMV infection occurs in about 1% of children born in the USA, resulting in approximately 40 000 new infections each year (Pass and Burke, 2002; Plotkin, 1999). More than 90% of infected infants are asymptomatic at birth, and most will escape serious consequences of HCMV infection. However, even among initially asymptomatic children, 5%–15% will eventually develop sequelae of infection including hearing loss, mental retardation, chorioretinitis or cerebral palsy (Fowler *et al.*, 1992; Revello and Gerna, 2002; Pass and Burke, 2002; Stagno *et al.*, 1982, 1986). Children born with symptomatic HCMV disease have a substantially worse prognosis. Approximately 10% will die, and most of the survivors will display profound deficits as the result of central nervous system damage (Istas *et al.*, 1995; Pass and Burke, 2002). All together, 4000 to 8000 children in the USA develop HCMV-related neurological disease each year, making HCMV the leading infectious cause of congenital mental retardation and deafness (Fowler *et al.*, 1992; Plotkin, 1999).

Despite the availability of antiviral therapy, HCMV disease also remains a feared complication in persons undergoing immunosuppressive therapy for malignancies or organ transplantation, and in persons with AIDS. In these individuals, who have severely impaired cellular immunity, HCMV can affect almost any organ system, and it commonly causes pneumonia, retinitis, hepatitis and ulcerative lesions of the gastrointestinal tract (Pass, 2001). For example, HCMV causes symptomatic illness in 35%, and death in 2% of all renal transplant recipients (Adler, 1996). In hematopoietic stem cell transplant (HSCT) recipients, the most common disease manifestation of HCMV infection is interstitial pneumonia (Leather and Wingard, 2001). Historically, this disease typically occurs during the first 100 days following transplantation. However, late-onset HCMV pneumonia is becoming more common due to the use of HCMV antiviral prophylaxis or pre-emptive therapy during the first 3 months following HSCT (Boeckh *et al.*, 1996; Leather and Wingard, 2001).

The case for a cytomegalovirus vaccine

Recognition of HCMV disease as a major public health problem has grown in the medical and scientific communities, if not among the general public. HCMV infection causes more CNS disease than did either *Hemophilus*

influenzae b or congenital rubella prior to their near eradication in the USA through vaccination (Pass and Burke, 2002). Moreover, it has been observed that HCMV infection now causes as many cases of mental retardation as the common genetic syndromes, trisomy 21 and fragile X chromosome (Plotkin, 1999). The disease burden associated with cytomegalovirus infection is estimated to cost the US healthcare system at least 4 billion dollars annually, with the majority of the cost attributable to long-term sequelae experienced by individuals who acquire congenital HCMV disease. These data placed HCMV in the highest priority grouping of vaccine targets in a recent Institute of Medicine report (Stratton *et al.*, 2000).

One alternative to vaccination, prevention of HCMV infection through public health measures, is complicated by the high prevalence of HCMV, its persistence following primary infection, its many avenues of transmission (including blood, urine, saliva, semen, breast milk, donated organs) and its propensity to be shed for long periods of time following primary infection, especially by children (Pass, 2001). Of particular relevance, non-pregnant women evaluated in a randomized, controlled trial who were given explicit instructions on methods to avoid HCMV infection nonetheless acquired HCMV infection at rates equivalent to women who received no counseling (Adler *et al.*, 1996).

The successful prevention and treatment of HCMV disease in immunocompromised patients has greatly improved in recent years due to continuing refinements in prophylactic and preemptive therapy for high risk individuals as well as an expanding arsenal of antiviral agents. Despite this, as many as 50% of solid organ transplant recipients develop symptomatic HCMV disease resulting in significant morbidity (Fishman and Rubin, 1998; Patel and Paya, 1997; Sia and Patel, 2000; Simon and Levin, 2003), and even with optimal therapy, mortality from HCMV pneumonia in hematopoietic stem cell transplant recipients may exceed 40% (Leather and Wingard, 2001). Recent data also suggest that antiviral therapy offers some benefit to newborns with HCMV disease (Michaels *et al.*, 2003; Kimberlin *et al.*, 2003). However, treatment alone will undoubtedly benefit only a minority of infants with congenital HCMV disease. Indeed, most infants who ultimately develop sequelae of HCMV infection are asymptomatic at birth and, thus, would not be considered for treatment (Griffiths, 2002). Also, treatment requires long-term antiviral therapy, which carries a substantial risk of complications, and treatment after birth is unlikely to repair organ damage, especially to the central nervous system, that occurred in utero (Kimberlin *et al.*, 2003; Michaels *et al.*, 2003). For all these reasons, then, a vaccine that prevents infection with HCMV, or at least mitigates its effects in vulnerable persons, is essential for eradicating the often devastating disease caused by HCMV.

Natural immunity confers protection

When considering the feasibility and design of an HCMV vaccine, it is important to first establish that natural immunity prevents disease. This issue is complicated for HCMV because virus capable of causing disease may arrive through three distinct avenues: primary infection, reactivation of virus already residing within the host, or reinfection of a previously infected individual with a different strain of HCMV. Data directly addressing the protective efficacy of pre-existing immunity in healthy adults is sparse. However, compelling evidence that previous infection prevents reinfection comes from a study of mothers of children shedding HCMV (Adler *et al.*, 1995). During the course of this study, 9 of 19 (47%) seronegative women developed primary infection, whereas only 3 of 42 (7%) seropositive women showed evidence of new HCMV infection, indicating 85% protection attributable to prior immunity (Plotkin, 2002). In addition, healthy adults with pre-existing immunity to HCMV were significantly protected from HCMV disease compared to seronegative individuals when challenged with the non-attenuated Toledo strain of HCMV (Plotkin *et al.*, 1989; Quinnan *et al.*, 1984). In this case, seropositive persons were also protected from HCMV infection, albeit to a lesser degree.

Since the primary goal of HCMV vaccination is to prevent congenital HCMV disease, the protection offered by preconceptual maternal immunity should predict the potential value of vaccination in this setting. Preconceptual immunity could protect newborns by preventing transmission of the virus to the fetus or by mitigating the effects of infection. Data indicate that maternal immunity to HCMV prior to conception provides both of these elements of protection. Primary infection during pregnancy results in transmission of HCMV to the fetus 15%–40% of the time, whereas women with preexisting immunity transmit HCMV only about 1%–2% of the time (Stagno *et al.*, 1982; Plotkin, 2002). In accordance with these data, a recent study showed that women who have naturally acquired immunity to HCMV prior to conception are 69% less likely to give birth to an infant with congenital HCMV infection than women who are initially seronegative (Fowler *et al.*, 2003). Moreover, congenital HCMV infections in infants born to women with HCMV immunity at the time of conception are considerably less likely to cause symptomatic disease at birth (Fowler *et al.*, 1992; Stagno *et al.*,

1982, 1997). In general, these children also have both fewer and less severe sequelae of HCMV infection, even when considering that adverse outcomes, such as hearing loss and mental retardation, may become apparent only months or years later (Fowler *et al.*, 1992). Maternal immunity prior to conception, however, does not confer complete protection against HCMV transmission to the fetus. Of note, a recent report concluded that while the severity of hearing loss in HCMV congenitally infected children was less if their mothers had preexisting immunity, the incidence of hearing loss was unaffected by maternal serostatus (Ross *et al.*, 2006). Indeed, in populations with high rates of HCMV seropositivity the majority of HCMV infections may occur in infants born to seropositive mothers even given the relatively low risk of transmission (Demmler, 1991; Pass and Burke, 2002; Stagno *et al.*, 1977; Schopfer *et al.*, 1978; Plotkin, 2002; Boppana *et al.*, 2001). Many of these infections presumably occur through the transmission of reactivated maternal virus, although a significant proportion undoubtedly arise from reinfection of the mother with a different strain of HCMV followed by transmission of the new virus to the fetus (Marshall and Plotkin, 1993; Boeckh *et al.*, 1996; Stagno *et al.*, 1982; Boppana *et al.*, 2001). In addition, symptomatic HCMV disease has been well documented in children born to mothers with preconceptual immunity to HCMV (Boppana *et al.*, 1999, 2001; Schopfer *et al.*, 1978; Ahlfors *et al.*, 1999; Morris *et al.*, 1994).

In conclusion, prior immunity to HCMV provides substantial protection against HCMV infection and disease, with the degree of protection estimated to be between 70% and 90% (Adler *et al.*, 1995; Fowler *et al.*, 2003; Plotkin, 2002). This conclusion engenders optimism that vaccination of seronegative girls prior to pregnancy may prevent a substantial proportion of cases of congenital HCMV disease and the attendant early and late sequelae. Moreover, vaccination may ultimately reduce asymptomatic infection and shedding by young children, which would lessen the reservoir of virus available to infect the fetuses of other mothers (Adler, 1988; Pass *et al.*, 1986). Indeed, it has been calculated that a vaccine that is only 60% effective against primary infection would be sufficient to eradicate HCMV from a given community within a developed country (Griffiths *et al.*, 2001).

Immunology of HCMV protection

Both neutralizing antibodies and cell-mediated immunity contribute to protection against HCMV disease (Table 71.1; for review see Gonczol and Plotkin, 2001; Plotkin, 2002; Pass and Burke, 2002). The importance of antibodies in preventing HCMV disease was first suggested by the observation that serious HCMV disease in newborn blood transfusion recipients was less frequent in infants born to seropositive mothers (Yeager *et al.*, 1981). This protection was presumably due to antibodies that had been transferred from the mother to the infant prior to birth. The protective benefit of HCMV antibodies in neonates was reinforced by subsequent data showing that passively administered antibodies, in the form of HCMV immune globulin, protected premature infants from HCMV disease (Snydman *et al.*, 1995). In addition, a nonrandomized study suggested that administration of HCMV-specific hyperimmune globulin to pregnant women may be effective in the treatment and prevention of congenital HCMV infection (Nigro *et al.*, 2005). HCMV immune globulin also appears to offer renal, liver, heart and bone marrow transplant recipients some protection from the most severe effects of HCMV disease (Snydman, 1993; Falagas *et al.*, 1997; Valantine, 1995; Bowden *et al.*, 1986; Glowacki and Smaill, 1994; Messori *et al.*, 1994). Protective levels of antibodies have not been established, but some evidence suggests that higher levels of neutralizing antibodies correlate with a lower risk for reinfection (Adler *et al.*, 1995). Regardless of the clinical setting, however, antibodies alone offer only partial protection and appear to be more effective in mitigating serious HCMV disease than in preventing infection.

Analysis of the HCMV genomic sequence suggests that is capable of encoding in excess of 60 glycoproteins, although how many are actually expressed is unknown (Chee *et al.*,

Table 71.1. Known targets of human immune responses to HCMV

HCMV gene product	Immune response
Glycoproteins	
gB	Major target of neutralizing antibodies; target of CTLs[a]
gH	Important target of neutralizing antibodies; target of CTLs
gM-gN	Target of antibody responses
US2, US3, US6, UL18	Targets of CTLs
Non-structural proteins	
pp65	Major target of CTLs; target of antibody responses
IE1	Important target of CTLs; target of antibody responses
pp150, pp28	Target of CTLs and antibody responses
pp50	Target of CTLs
pp71, pp52	Targets of antibody responses

[a] Cytotoxic T-lymphocytes.

1990; Cha *et al.*, 1996; Davison *et al.*, 2003). Most HCMV neutralizing antibodies, however, appear to recognize a tiny subset of these proteins, namely, glycoprotein B (gB), glycoprotein H (gH) and the glycoprotein M-N (gM-gN) complex (Britt *et al.*, 1990; Kari and Gehrz, 1990; Mach *et al.*, 2000; Marshall *et al.*, 1992, 1994; Rasmussen *et al.*, 1991; Urban *et al.*, 1996).

Glycoprotein B is the most abundant membrane protein in the HCMV envelope (Britt and Mach, 1996). It is highly conserved among all mammalian herpesviruses, and participates in several facets of the virus life cycle including entry and cell–cell spread (Bolds *et al.*, 1996; Compton *et al.*, 1993; Navarro *et al.*, 1993). Recently, it was shown that HCMV entry into cells is mediated by gB binding to the cellular epidermal growth factor receptor (Wang *et al.*, 2003). HCMV gB, like the gB homologues in other herpesviruses, is a large type I membrane protein (for review see Britt and Mach, 1996; Spaete, 1994). It is cleaved by a host cell protease into two peptides, which remain disulfide-linked. Glycoprotein B is modified by N- and O-glycosylation and forms homodimers in both virions and infected cells (Mocarski and Courcelle, 2001). Glycoprotein B appears to be the most immunogenic HCMV protein. Almost all persons develop antibodies to gB following HCMV infection, and gB-specific antibodies account for 40%–70% of the total HCMV neutralizing activity in HCMV seropositive individuals (Britt *et al.*, 1990; Cremer *et al.*, 1985; Kniess *et al.*, 1991; Marshall *et al.*, 1992). Glycoprotein B contains two well-characterized major antigenic domains, AD-1 and AD-2, that are capable of inducing neutralizing antibodies during infection (Britt *et al.*, 1988; Kniess *et al.*, 1991; Marshall *et al.*, 2000; Meyer *et al.*, 1992; Wagner *et al.*, 1992). The antibody response after natural infection is directed most frequently to AD-1, which is highly conserved in clinical isolates (Schoppel *et al.*, 1996; Wada *et al.*, 1997; Chou and Dennison, 1991). However, 11% of HCMV seropositive persons lacked antibodies to linear epitopes on either AD-1 or AD-2, but had neutralizing activity suggesting that for some individuals, different epitopes in gB or epitopes in other HCMV proteins may be more important in the generation of virus neutralizing responses (Marshall *et al.*, 2000).

Glycoprotein H is a relatively abundant component of the virion envelope and is also conserved among the mammalian herpesviruses, although it is much more divergent than gB (Mocarski and Courcelle, 2001). HCMV gH has been shown to participate in membrane fusion, and it may play role in virus entry at a step following attachment (Britt and Mach, 1996; Mocarski and Courcelle, 2001; Keay and Baldwin, 1991; Rasmussen *et al.*, 1984). Glycoprotein H is also an important target of host immune responses, and almost all persons infected with HCMV develop gH-specific antibodies (Urban *et al.*, 1996). In some cases, gH may be the dominant target of neutralizing antibodies as it has been shown to account for 0–58% of the total virus neutralizing activity in persons with a past history of HCMV infection (Urban *et al.*, 1996). Like gB, antigenic domains have been identified in gH (Simpson *et al.*, 1993). Interestingly, HIV-infected persons with CD4 counts less than 100 cells/mm^3 who had histories of past HCMV infections rarely had detectable gH antibody titers compared to persons with higher CD4 counts, while gB titers were unaffected by the CD4 count (Rasmussen *et al.*, 1994). Given that HIV-infected persons with CD4 counts less than 100 cells/mm^3 are at high risk for retinitis due to reactivated HCMV, this finding raises the possibility that gH antibodies may be necessary for containing reactivated virus in some settings.

Recently, the gM-gN complex was recognized as an important target of antibody responses in seropositive adults (Kari and Gehrz, 1990; Mach *et al.*, 2000). Sera from HCMV-infected adults failed to recognize gM or gN when they were expressed alone; however, sera from 62% of previously infected individuals reacted with the gM–gN complex. The importance of gM–gN antibodies in preventing HCMV disease is unknown. A possible link between gM–gN antibodies and human disease, however, was suggested by the observation that the 14 of 16 congenitally infected infants lacked detectable antibodies against this complex, whereas most adults in the same study had gM–gN antibodies (Mach *et al.*, 2000).

In addition to gB, gH and gM–gN, antibodies to several non-envelope HCMV proteins, including pp65, IE1, pp150, pp28, pp71 and pp52, are commonly detected in seropositive people (Pass and Burke, 2002). It remains to be determined whether these antibodies contribute to protection against HCMV infection and disease.

The pivotal role for cell-mediated immunity in the control of HCMV infection is underscored by the fact that virtually all cases of severe HCMV disease not associated with congenital infection occur in persons with profoundly impaired cellular immunity, and the severity of HCMV disease typically correlates with the degree of immunosuppression. Specifically, HLA-restricted CD8$^+$ cytotoxic T lymphocyte (CTL) responses are crucial for the control of HCMV disease in immunocompromised persons (Quinnan *et al.*, 1982; Li *et al.*, 1994; Reusser *et al.*, 1991). Allogeneic marrow transplant recipients are at high risk for HCMV disease until their CD8$^+$ CTLs return. The fundamental importance of CTL responses in controlling HCMV disease was directly assessed by an adoptive transfer study in which marrow transplant recipients received serial transfusions of HCMV-specific CTLs (Walter *et al.*, 1995). None of the 14 very high-risk patients in this study developed HCMV

Table 71.2. Status of HCMV vaccines currently being tested

	Stage of testing			
		Human trials		
Vaccine	Preclinical	Safety	Immunogenicity	Efficacy
Towne vaccine	+	+	+	+
gB subunit vaccines	+	+	+	+[a]
ALVAC-based vaccines	+	+	+	−
Towne/Toledo chimeric vaccines	+	+	−	−
DNA vaccines	+	−	−	−
Peptide vaccines	+	−	−	−
Dense body vaccines	+	−	−	−

[a] This study is ongoing, and no efficacy data are yet available.

viremia or disease. Therefore, considerable effort has been devoted in recent years to identifying the HCMV targets of CTL responses since the induction of such responses may be imperative for the success of an HCMV vaccine. This work led to the discovery that the tegument protein pp65 is the dominant target of virus-specific CTLs. In persons with past HCMV infection, approximately 70–90% of all CTLs that recognize HCMV-infected cells are specific for this protein (Boppana and Britt, 1996; Kern *et al.*, 2002; McLaughlin-Taylor *et al.*, 1994; Wills *et al.*, 1996). Recently, specific peptides derived from the pp65 sequence have been identified that are able to induce HCMV-specific CTL in an HLA-A24-restricted manner (Akiyama *et al.*, 2002; Masuoka *et al.*, 2001).

While it is striking that a single protein induces so much of the CTL response directed against such a complex virus, other HCMV proteins have also been shown to contain CTL epitopes. Most notable among these are the HCMV immediate-early protein, IE1, and the tegument protein, pp150, which in some individuals appears to induce CTL responses with similar precursor frequencies to pp65 (Kern *et al.*, 2000; Gyulai *et al.*, 2000; La Rosa *et al.*, 2005). Like pp65, IE1- and pp150 derived peptides that induce CTL in an HLA-restricted manner have been identified (Frankenberg *et al.*, 2002; La Rosa *et al.*, 2005). In addition, other HCMV antigens that are capable of inducing CTL responses including gB, gH, pp150, pp28, pp50, US2, US3, US6, and US18 (Boppana and Britt, 1996; Gyulai *et al.*, 2000; Elkington *et al.*, 2003).

The effectiveness of an HCMV vaccine is likely to be enhanced by, and may absolutely require, the induction of HCMV-specific CTL responses. Such responses would be expected following inoculation with live attenuated vaccines. However, other vaccine approaches, such as vectored vaccines and DNA vaccines, as discussed below, may require the inclusion of specific CTL epitopes to achieve similar results. Moreover, multiple CTL epitopes may need to be included to ensure maximal coverage in the community at large.

Vaccine development

HCMV has been the target of active vaccine development efforts since the 1970s. Early work focused on the development of live-attenuated vaccines, which have now been tested in numerous human trials and, as a family, continue to show considerable promise. With advances in molecular techniques, and rapidly expanding knowledge of HCMV biology and immunology, several other approaches are currently being applied to the development of safe and effective HCMV vaccines (Table 71.2).

Replicating vaccines

The first HCMV vaccine tested in humans was AD169, a laboratory-adapted strain of HCMV made by passaging virus isolated from human adenoidal tissue a total of 54 times in four different cultured human fibroblast cell lines (Elek and Stern, 1974). A lysate containing infectious virus derived from sonicated AD169-infected cells was administered to HCMV seronegative adults. Twenty-five of 26 volunteers (96%) who received 10 000 plaque forming units (pfu) of virus subcutaneously seroconverted. The vaccine was safe and well tolerated with 12 of 26 recipients exhibiting minor local reactions and one person developing lymphadenopathy and lymphocytosis. None of the vaccinees excreted virus based on cultures of throat washings and

urine. Two vaccinees tested a year later showed no reduction in antibody titers. However, evaluation of some of these subjects 8 years later revealed that only half had detectable HCMV antibody or lymphocyte transformation responses (Stern, 1984).

A second clinical trial of AD169 was conducted a few years after the first (Neff *et al.*, 1979). The virus used in this trial was passaged an additional five times and prepared as a filtered sonicate of infected cells. Twenty-four adult men were vaccinated subcutaneously, and all 20 of the initially seronegative individuals developed antibodies to HCMV by one month following vaccination. One year later, immune adherence antibodies had declined slightly while complement-fixing antibodies had declined significantly. Participants with pre-existing immunity to HCMV exhibited no antibody response to vaccination. The vaccine virus could not be detected in leukocytes, urine or throat specimens from the vaccinated seronegative persons and was not transmitted to any of the 10 seronegative contacts of the vaccine recipients, which was taken as evidence for lack of contagiousness of the vaccine virus. AD169 was not pursued further as a vaccine candidate. Instead, attention turned to the HCMV Towne strain as a potential live attenuated vaccine, and Towne remains today the best-studied HCMV vaccine candidate.

The Towne strain of HCMV was isolated in 1970 from the urine of a 2-month old infant with congenital disease, then passaged 125 times exclusively in WI-38 human diploid fibroblasts, including three clonings (Plotkin *et al.*, 1975). It was characterized in 1975 prior to its use in clinical trials and was shown to possess several characteristics that distinguished it from native HCMV indicating that it had been altered by cell culture passage. These included increased production of cell-free virus, thermostability and trypsin resistance. In addition, safety tests in various animals and cell lines showed the virus stocks to be free of adventitious agents.

In the first published human trial describing Towne vaccination, all 10 seronegative adults inoculated intramuscularly seroconverted within four weeks while 11 seronegative adults inoculated both intranasally and orally failed to seroconvert (Just *et al.*, 1975). Five persons with pre-existing immunity to HCMV were vaccinated with Towne and showed no increase in antibody titers. None of the participants had systemic symptoms or atypical lymphocytosis; however, 7 of the original 10 seronegative vaccinees developed mild local reactions beginning 14–16 days following vaccination and lasting about a week. As with the AD169, Towne could not be isolated from the leukocytes or urine of any vaccinee, suggesting that Towne is attenuated and unable to persist in vaccinated persons.

A second early trial with Towne largely reinforced the findings from the first trial (Plotkin *et al.*, 1976). Once again, persons inoculated intranasally failed to seroconvert whereas all seronegative volunteers who were vaccinated subcutaneously acquired HCMV-specific antibodies, and no excretion of vaccine virus from these individuals could be documented. In contrast to the earlier study, however, all 4 seronegative participants in this trial acquired complement-fixing antibodies compared to 1 of 10 in the first trial. Also, both initially seropositive vaccinees developed IgM antibodies, raising the possibility that Towne may reinfect persons who had previously been infected with native HCMV.

Subsequent human trials with Towne confirmed its ability to elicit both binding and neutralizing antibodies, and demonstrated that Towne-induced antibodies have similar specificities to the antibodies arising from natural HCMV infection (Gonczol *et al.*, 1989). Early studies showed that both binding and neutralizing antibody titers in response to Towne vaccination were substantially lower than those following natural infection (Gonczol *et al.*, 1989; Adler *et al.*, 1995). However, a more recent study showed that a new lot of Towne vaccine induced titers of neutralizing antibodies comparable to those induced by natural infection and that the response was dose dependent (Adler *et al.*, 1998). In this study, as in earlier trials, the level of Towne-induced antibodies waned over the course of a year, while those in naturally seropositive women remained stable.

The ability of Towne to induce cellular immune responses is well documented. Towne vaccination of healthy seronegative adults uniformly results in HCMV-specific lymphocyte responses, a surrogate for CD4+ cell activation, which persist for at least 10 months (Gehrz *et al.*, 1980; Starr *et al.*, 1981; Fleisher *et al.*, 1982; Plotkin *et al.*, 1989; Adler *et al.*, 1995, 1998). In addition, Towne consistently elicits HCMV-specific CD8+ CTL responses in immunocompetent individuals (Quinnan *et al.*, 1984; Adler *et al.*, 1998).

Towne has also been tested in a series of studies in prospective kidney transplant recipients, a population that is at high risk for HCMV disease following transplantation (Glazer *et al.*, 1979; Marker *et al.*, 1981; Starr *et al.*, 1981; Plotkin *et al.*, 1984, 1991, 1994; Brayman *et al.*, 1988; Balfour, 1991). Most renal transplant candidates developed humoral and cellular immune responses following Towne vaccination. The responses, however, tended to be delayed or diminished when compared to those of healthy vaccinees (Starr *et al.*, 1981; Plotkin *et al.*, 1984, 1991). Three controlled trials have been conducted in which renal transplant candidates received either Towne vaccine or a placebo (Balfour, 1991; Plotkin *et al.*, 1991, 1994). Each of

Table 71.3. Protective efficacy of Towne vaccination in seronegative renal transplant recipients who received kidneys from seropositive donors

Trial	*n* (vaccine/ placebo)	All HCMV disease		Severe HCMV disease		% reduction of all disease	% reduction of severe disease
		Vaccine	Placebo	Vaccine	Placebo		
Plotkin *et al.*, 1991	67 (36/31)	14 (39%)	17 (55%)	2 (6%)	11 (35%)	46%	83%
Balfour 1991	35 (21/14)	8 (38%)	6 (43%)	2 (10%)	5 (36%)	12%	72%
Plotkin *et al.*, 1994	61 (37/24)	14 (38%)	14 (59%)	0 (0%)	4 (17%)	36%	100%
All	**163 (94/69)**	**36 (38%)**	**37 (54%)**	**4 (4%)**	**20 (29%)**	**30%**	**86%**

these trials yielded similar results (Table 71.3). Vaccination with Towne failed to prevent HCMV infection following transplantation, and while the incidence of total HCMV disease was decreased, this effect did not achieve statistical significance. However, in the highest risk population, namely HCMV seronegative persons who received a kidney from a seropositive donor, the incidence of severe HCMV disease was reduced by 72%–100%, a degree of protection comparable to that stimulated by natural infection (Plotkin *et al.*, 1991).

The efficacy of Towne vaccination was also assessed in a controlled challenge study. In that study, seronegative adults were vaccinated with Towne and challenged with the non-attenuated, low-passage Toledo strain of HCMV (Plotkin *et al.*, 1989). The ability of Toledo to cause disease was confirmed when all 6 seronegative, unvaccinated persons challenged subcutaneously with 10 or 100 pfu of Toledo developed clinical symptoms of HCMV disease as well as evidence of infection based positive viral cultures from their blood, urine and/or saliva. Twelve adults who had been vaccinated with Towne 1 year earlier were challenged with Toledo. All 5 who were challenged with 10 pfu of Toledo were protected from infection and disease. However, of the 7 Towne-vaccinated persons who were challenged with 100 pfu of Toledo, most (4/7) showed evidence of infection based on virus culture, one had clinical illness and 3 had laboratory abnormalities suggestive of HCMV infection. In contrast, naturally seropositive persons were protected from challenge with 100 pfu of Toledo; however, 5/5 seropositive individuals challenged with 1000 pfu of Toledo exhibited clinical disease, laboratory abnormalities and/or positive HCMV cultures. Therefore, in this small study, Towne appeared to afford some protection against HCMV infection and disease, but less than natural infection. This study and an earlier challenge study in seropositive, unvaccinated individuals (Quinnan *et al.*, 1984) provided valuable information not only on the protective efficacy of Towne relative to natural infection, but also on the dose dependence and natural history of HCMV disease in immunocompetent adults. However, challenge studies with HCMV may no longer be possible given contemporary regulatory standards designed to ensure volunteer safety.

To assess the protective efficacy of Towne in a more natural setting, a placebo-controlled study was performed in seronegative women with children in daycare. This was, in effect, a challenge study as the parents of children in daycare are at high risk for HCMV infection (Pass *et al.*, 1986). In this population, Towne vaccination failed to protect women from HCMV infection, while natural infection was highly protective against re-infection with HCMV (Adler *et al.*, 1995).

Towne remains the only HCMV vaccine candidate that has completed efficacy testing in any human population. In the final estimation, Towne vaccination induces both humoral and cellular immunity, and it is capable of providing some protection against HCMV disease in certain settings. However, it is clearly less protective than natural infection, particularly in its ability to prevent infection with native HCMV, which may be critical for preventing congenital disease. In an effort to enhance the immunogenicity of Towne, the co-administration of recombinant human IL-12 with Towne was evaluated in a recent clinical trial (Jacobson *et al.*, 2006). This combination proved to be well tolerated and resulted in enhanced HCMV-specific antibody and T cell responses.

The nature of the deficit in Towne's ability to stimulate protective immunity is unknown, but it has been suggested that the lower neutralizing antibody titers induced by Towne compared to natural infection may be at fault (Wang *et al.*, 1996). Regardless, the experience with Towne suggested that it is overly attenuated and prompted researchers to pursue less attenuated live vaccines that will, ideally, retain the excellent safety and tolerability profiles of Towne.

The high level of attenuation exhibited by Towne is presumably due to genetic mutations introduced during its extensive passage in cultured cells (Huang *et al.*, 1980; Prichard *et al.*, 2001). Furthermore, recent data indicate

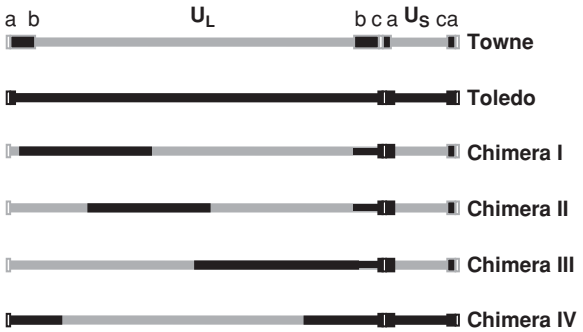

Fig. 71.1. Genomic structures of the two parental strains and four chimeras. The specific regions of each chimera's genome derived from Towne(▬) and Toledo(▬) are shown. The UL/b' region(▬) of Toledo is marked(courtesy of George Kemble)

that HCMV Towne contains numerous mutations throughout in its genome when compared to the non-attenuated Toledo strain of HCMV (G. Kemble, personal communication, 2003); however, the specific mutations causing attenuation are not known. In an effort to produce a vaccine that is intermediate in attenuation between HCMV Towne and wild-type virus, genetic recombinants were constructed in which regions from the HCMV Toledo genome were substituted for the corresponding regions of the Towne genome using cosmid-based mutagenesis (Kemble *et al.*, 1996). Four independent chimeric viruses (referred to as Chimeras I–IV) were produced in which every region of the Towne genome was replaced sequentially by Toledo sequences (Fig. 71.1). Each of the four chimeric vaccine candidates made using this approach contains the UL/b region of the HCMV genome. This region, which is predicted to encode 19 proteins, is universally found in the genomes of circulating HCMV isolates but is not present in it entirety in many HCMV strains that have undergone extensive passage in cell culture, such as AD169 (Cha *et al.*, 1996). It was hypothesized that by using this approach, one or more of the Towne/Toledo recombinants would contain some, but not all, of the mutations that confer attenuation on Towne. This, in turn, should result in a chimeric virus that is attenuated relative to Toledo, but less attenuated than Towne.

The four Towne/Toledo recombinant viruses were evaluated in a recently completed double-blinded, placebo controlled clinical trial (Heineman *et al.*, 2003). The study was designed to determine whether the vaccine candidates are safe and well tolerated, whether they are attenuated relative to Toledo and whether they are shed in the blood, urine or saliva of vaccinees. Healthy HCMV seropositive adults each received a single dose of 1000 pfu of one of the four investigational vaccines or an inactive placebo. Participants were evaluated weekly for 8 weeks, then less frequently for the remainder of a year, for clinical or laboratory evidence of HCMV infection and disease. All four vaccine candidates were safe and well-tolerated although as a group they produced more local reactogenicity than the placebo. As predicted, each of the Towne/Toledo chimeric vaccine candidates was attenuated relative to Toledo based on comparison to the previous Toledo challenge data discussed above (Quinnan *et al.*, 1984; Plotkin *et al.*, 1989). This attenuation was evident from the paucity of laboratory abnormalities suggestive of HCMV infection in the recipients of the chimeric vaccine candidates in contrast to those noted in HCMV seropositive persons who had received the same dose of Toledo (Table 71.4). However, the degree of attenuation of the four Towne/Toledo chimeras relative to each other could not be discerned from this initial trial. Like Towne, none of the vaccine candidates was cultured from the blood, urine or saliva of any vaccinee or any of their close contacts suggesting that systemic infection did not occur in this population. Immunogenicity data from this trial are pending. Future studies are planned to address the safety and immunogenicity of these vaccine candidates in seronegative persons, who comprise the target population for vaccination.

Table 71.4. Towne/Toledo chimeric vaccine candidates are attenuated relative to Toledo in natural seropositives inoculated with 1000 pfu

Vaccine	Laboratory abnormalities[a]	Virus isolated
Chimera I	0/5	0/5
Chimera II	1/5[c]	0/5
Chimera III	0/5	0/5
Chimera IV	1/5[d]	0/5
Chimeras I-IV	**2/20**	**0/20**
Placebo	1/5[c]	0/5
HCMV Toledo[b]	***5/5***	***2/5***

[a] Defined as elevated aspartate aminotransferase (AST) or atypical lymphocytosis.
[b] From Plotkin, *et al.*, 1989.
[c] Elevated AST. In the case of Chimera II, the volunteer had an elevated AST prior to vaccination.
[d] Atypical lymphocytosis of 9% at week 8 after vaccination only.

Subunit vaccines

Subunit vaccines, in which a single or a few specific proteins are used in combination with an adjuvant to

stimulate protective immunity, are attractive for several reasons. Most importantly, they are not infectious and contain no genetic material thus eliminating some safety concerns. Also, using modern molecular genetic methods, large amounts of the vaccine antigens can be produced easily and cheaply. Finally, subunit vaccines for numerous infectious diseases have been studied extensively in animals and, in some cases, have progressed to human trials. Of particular relevance, recent data suggest that a subunit vaccine derived from a single herpes simplex virus (HSV) type 2 protein may protect seronegative women from disease caused by primary HSV infection (Stanberry *et al.*, 2002).

As discussed above, HCMV gB is both highly immunogenic and highly conserved between HCMV isolates. For these reasons, and because it is the best studied HCMV glycoprotein, gB has been the primary antigen used in most HCMV subunit vaccine studies. Glycoprotein B used in vaccine studies has been modified to facilitate its purification. To that end, its hydrophobic transmembrane domain has been removed, and it has been mutated to eliminate its internal proteolytic cleavage site. Thus, the form of gB used in human trials is expressed in Chinese hamster ovary cells and is purified as an excreted protein consisting of a single 807 amino acid peptide (Pass *et al.*, 1999).

The choice of vaccine adjuvants has considerable impact on the immunogenicity of subunit vaccines. In the human trials reported to date, HCMV gB has been combined with either aluminum hydroxide (alum), the adjuvant used in the licensed hepatitis B vaccines, or MF59, a proprietary oil-in-water emulsion of squalene (Chiron Vaccines, Emeryville California) (Ott *et al.*, 1995). MF59 was previously shown to induce higher antibody titers than alum when combined with a variety of viral antigens (McElrath, 1995). Accordingly, virtually all healthy seronegative adults inoculated at 0, 1 and 6 months with HCMV gB combined with MF59 developed levels of binding and neutralizing antibodies comparable to those induced by natural infection, whereas persons vaccinated similarly with gB/alum produced significantly lower titers of gB-specific antibodies (Pass *et al.*, 1999). Also, IgG and IgA antibodies to gB were present in the saliva or nasal washes of most gB/MF59 recipients (Wang *et al.*, 1996). Dose comparison studies demonstrated that low doses of gB (5 μg) combined with MF59 elicited antibody responses similar to those observed with higher doses (30 or 100 μg) (Pass *et al.*, 1999; Frey *et al.*, 1999). Toddlers who received three 20 μg doses of gB/MF59 developed mean gB binding and neutralizing antibody titers six-fold higher than were observed in earlier adult studies (Mitchell *et al.*, 2002). Following vaccination with gB/MF59, neutralizing antibody titers waned rapidly, and it was suggested that insufficient $CD4^+$ responses might have contributed to this decline. Nonetheless, neutralizing antibody titers rebounded dramatically after an additional dose of vaccine (Plotkin, 2001; Pass and Burke, 2002). Vaccination with gB/MF59 also induced strong lymphocyte proliferative responses to both gB and HCMV, and these declined little during the year following vaccination (Pass and Burke, 2002). The HCMV subunit vaccines caused more injection site pain than placebo; however, both were generally well tolerated.

The studies described above laid the groundwork for efficacy trials of the gB/MF59 subunit vaccine. Currently, the ability of gB/MF59 to prevent congenital HCMV infection in the children of healthy seronegative women is being assessed in a double-blinded, placebo controlled trial (R. Pass, personal communication, 2006). In addition, a study is being planned to determine whether gB/MF59 vaccination protects adolescents from HCMV infection, a population that may ultimately be targeted for vaccination (D. Bernstein, personal communication, 2003). While HCMV subunit vaccines have thus far focused on the use of gB as the immunogen, future subunit vaccines may include gH, gM-gN, and perhaps other HCMV antigens.

Vectored vaccines

A number of viruses have been utilized as vectors to express potential vaccine antigens. Of these, the attenuated ALVAC strain of canarypox has been most extensively employed as a vector for the delivery of HCMV antigens (Baxby and Paoletti, 1992; Tartaglia *et al.*, 1992; Plotkin *et al.*, 1995). The ALVAC genome will accommodate the insertion of large exogenous DNA fragments providing great flexibility in the choice of antigen genes or combinations of genes. While it can infect human cells and express foreign antigens, its own genome is not replicated and progeny virions are not produced, thus reducing the risk of vaccine-associated complications. Most importantly from the perspective of vaccine efficacy, foreign antigens expressed by ALVAC are transported and processed authentically within cells allowing their presentation in the context of MHC class I molecules. This, in turn, may facilitate the stimulation of CTL responses that mimic those of natural infection (Pialoux *et al.*, 1995; Clements-Mann *et al.*, 1998; Taylor *et al.*, 1995; Plotkin *et al.*, 1995).

Because of its preeminence as target for neutralizing immunity, gB was the first HCMV antigen chosen for expression by ALVAC. Studies in mice and guinea pigs showed that two doses of ALVAC-gB induced neutralizing antibodies and also high levels of HCMV-specific $CD8^+$ CTL

responses (Gonczol *et al.*, 1995). UV-inactivated ALVAC-gB failed to induce CTLs indicating that de novo synthesis of gB was required. In addition, prior vaccinia virus exposure did not inhibit the gB-specific immunity induced by ALVAC-gB in mice, addressing an issue that may become more important if vaccinia vaccination rates increase in response to bioterrorism concerns. The promise of the initial animal studies, however, was not fully realized in early human trials. Even after three doses of ALVAC-gB, seronegative adults failed to develop significant HCMV neutralizing antibody titers perhaps reflecting low levels of antigen production (Adler *et al.*, 1999).

ALVAC-gB proved far more successful when it was used to "prime" the immune system in so-called prime-boost vaccination strategies. HCMV seronegative adults who were primed with two doses of ALVAC-gB at days 0 and 30, then boosted with a single dose of Towne at day 90 developed binding and neutralizing antibody titers at least as high as naturally seropositive individuals (Adler *et al.*, 1999). Similarly, individuals primed with two doses of ALVAC-gB, then boosted with gB/MF59 subunit vaccine developed high antibody titers and lymphoproliferative anti-HCMV responses. These humoral and cellular responses, however, were not significantly different from those elicited by three doses of gB/MF59 alone, thus demonstrating no clear benefit to priming with ALVAC-gB (Bernstein *et al.*, 2002).

The greatest value of canarypox-based vaccines may derive from their ability to stimulate specific CTL responses. ALVAC expressing pp65, an abundant tegument protein that is a major CTL target in naturally HCMV seropositive persons, induced $CD8^+$ CTL responses in all seronegative persons tested (Berencsi *et al.*, 2001). The CTL responses were detected after two doses of ALVAC-pp65 and were present at frequencies comparable to those seen in naturally seropositive individuals. Ultimately, canarypox-based vaccines that express both CTL and neutralizing antibody targets, such as pp65 and gB, may be used in prime-boost vaccination protocols. For example, two doses of an ALVAC-based vaccine may be followed by a boost with a subunit or live-attenuated vaccine, to confer high levels of both cellular and humoral immunity.

Peptide vaccines

The development of peptide vaccines for HCMV represents an effort to directly stimulate a protective CTL response. Toward this end, a 9 amino acid minimal cytotoxic epitope derived from HCMV pp65 was identified and lipidated at its amino terminus to allow its administration without an adjuvant (Diamond *et al.*, 1997; Martinon *et al.*, 1992). HLA A2.1 transgenic mice immunized with this peptide, in combination with the PADRE pan-HLA-DR T-helper epitope, developed HCMV-specific CTL. Subsequent studies showed that linking the HCMV pp65 epitope directly to the PADRE epitope in the same peptide elicited vigorous HCMV-specific CTL responses in HLA transgenic mice when delivered either subcutaneously or intranasally (La Rosa *et al.*, 2002). In either case, the response was significantly enhanced by the co-administration of CpG-containing single stranded DNA, which enhances the immune response and biases it in the direction of Th1 activity (Klinman, 2003). A repertoire of HCMV epitopes specific for different HLA alleles has been defined that should provide 90% coverage to the Caucasian population, although the derivation of four or more additional CTL epitopes would be needed to attain 90% coverage for African Americans or Asians (Longmate *et al.*, 2001). Peptide vaccination may have its primary utility in therapeutic rather than prophylactic vaccination as HLA allele-specific peptides may be limited in their ability to elicit immunity in the population at large (Gonczol and Plotkin, 2001). To that end, the HCMV-pp65-specific memory CTL response could be amplified in a hematopoietic stem cell donor prior to transplantation by administration of a peptide vaccine, thereby providing protection against HCMV disease in the recipient (La Rosa *et al.*, 2002).

DNA vaccines

The initially surprising discovery that direct injection of purified DNA encoding specific antigens can induce protective immunity led to the development of DNA vaccination strategies for many pathogens including HCMV (Wolff *et al.*, 1990; Ulmer *et al.*, 1993). In this approach, as with live attenuated and vectored vaccines, HCMV antigens are synthesized and processed using authentic cellular pathways thus allowing their expression in the context of MHC class I molecules (Tang *et al.*, 1992; Ulmer *et al.*, 1993; Raz *et al.*, 1994). This approach, therefore, has the potential to induce both humoral and cellular immunity to HCMV. While DNA vaccines for HCMV have yet to be studied in humans, compelling data, some of which is discussed below, has been generated in animal models.

The first DNA vaccine for HCMV consisted of a plasmid containing the gene for pp65 (Pande *et al.*, 1995). Most of the mice injected intramuscularly with this plasmid developed antibodies to pp65 confirming that vaccination with HCMV DNA was capable of eliciting an antigen-specific immune response. The protective efficacy of DNA vaccination was subsequently demonstrated in mice (Gonzales Armas *et al.*, 1996). After inoculation with the murine CMV

(MCMV) pp89 gene, the major target for $CD8^+$ T-cells (Reddehase and Koszinowski 1984; Holtappels *et al.*, 1998), mice exhibited 45% protection against lethal challenge as well as significantly lower viral titers in the spleen and salivary glands. A DNA vaccine expressing a different MCMV antigen, M84, which is homologous to HCMV pp65, also afforded some protection to mice. Coimmunization with both pp89 and M84 DNA vaccines provided the best protection suggesting that the most successful DNA vaccines may express multiple antigens (Morello *et al.*, 2000; Ye *et al.*, 2002).

Considerable evidence suggests that both humoral and cell-mediated immunity participate in the control of HCMV disease. Accordingly, DNA vaccines expressing gB were tested in mice and shown to elicit neutralizing antibodies (Hwang *et al.*, 1999). Therefore, a second generation of HCMV DNA vaccines was designed to stimulate both neutralizing antibodies to HCMV and HCMV-specific CTL responses. These vaccines consisted of a cocktail of plasmids encoding pp65, to stimulate CTL responses, and gB, to induce neutralizing antibodies (Endresz *et al.*, 1999; Schleiss *et al.*, 2000). After three immunizations, all mice developed gB- and pp65-specific antibodies, and about 60% developed pp65-specific CTL responses. Similarly, guinea pigs also developed antibodies to both antigens.

Recently, various innovative methods to enhance immune responses have been applied to HCMV DNA vaccines. Aluminum salts, which are licensed for use as adjuvants in humans, and CpG oligodeoxynucleotides have both been shown to enhance antibody responses to DNA vaccines (Ulmer *et al.*, 1999; Klinman *et al.*, 2000). A DNA vaccine containing the HCMV gB gene and administered with aluminum phosphate gel elicited significantly higher antibody responses in mice than the gB gene without aluminum phosphate gel, although no difference was seen in neutralizing antibody titers (Temperton, 2002; Temperton *et al.*, 2003). However, the addition of CpG oligodeoxynucleotides to aluminum phosphate gel enhanced the ability of the gB gene to stimulate neutralizing antibodies. Using a related approach, several laboratories have shown that coinoculation of animals with genes encoding immunostimulatory molecules in addition to viral antigens can provide enhanced protection against disease (Tsuji *et al.*, 1997; Xiang and Ertl, 1995; Cull *et al.*, 2002). Applying this approach, mice coimmunized with the MCMV gB and type I interferon genes exhibited enhanced protection against MCMV challenge when compared to mice that received the MCMV gB gene alone (Cull *et al.*, 2002). It is interesting to note that DNA vaccination with type I interferons alone also reduce the level of MCMV infection (Yeow *et al.*, 1998; Cull *et al.*, 2002; Bartlett *et al.*, 2002). Recently, the use of full-length murine and guinea pig CMVs cloned as bacterial artificial chromosomes (BACs) have been used as DNA vaccines. In principle, BAC vaccines would deliver the full complement of viral genes thereby inducing a wide range of antiviral immune responses (Cicin-Sain *et al.*, 2003; Schleiss *et al.*, 2006). Preconceptual maternal immunization of guinea pigs with a replication-disable guinea pig CMV BAC significantly protected their offspring against congenital CMV disease (Schleiss *et al.*, 2006).

DNA vaccines for HCMV are still in their infancy, and safety issues stemming from the inoculation of exogenous DNA still need to be resolved. Nonetheless, this approach is remarkably versatile and may ultimately have an important place in HCMV vaccine development.

Subviral particles

Upon infection with HCMV, cultured fibroblasts release not only infectious virions but also non-infectious particles (Craighead *et al.*, 1972; Fiala *et al.*, 1976; Sarov and Abady, 1975). These non-infectious particles may be either dense bodies, which are enveloped structures consisting of viral tegument proteins and glycoproteins but lacking a capsid, or non-infectious enveloped particles (NIEPs), which are similar to normal virions except for the absence of DNA and the presence of an additional polypeptide. Glycoprotein B and the tegument protein pp65 are major constituents of dense bodies, and sera from HCMV seropositive individuals react with these particles (Baldick and Shenk, 1996; Forghani and Schmidt, 1980; Gibson and Irmiere, 1984; Irmiere and Gibson, 1983; Roby and Gibson, 1986). For this reason, dense bodies have long been considered as possible HCMV vaccines (Bia *et al.*, 1980; Fiala *et al.*, 1976; Gibson and Irmiere, 1984; Sarov *et al.*, 1975; Stinski, 1976). More recent studies showed that dense bodies enter cells efficiently, presumably through the interaction of envelope glycoproteins with cellular receptors, thus mimicking normal infection (Pepperl *et al.*, 2000; Schmolke *et al.*, 1995; Topilko and Michelson, 1994). Mice transgenic for human HLA-A2 were immunized with dense bodies without an adjuvant. After only a single inoculation, the mice developed high virus neutralization titers, and those that received three doses retained substantial virus neutralizing activity for at least a year (Pepperl *et al.*, 2000). The immunized mice developed antibodies to gB, gH, pp65, pp150 and several unidentified proteins. Given the importance of cellular immunity in controlling HCMV disease, it is significant to note that the immunized mice also developed high levels of HCMV-specific CTLs despite the absence of HCMV protein synthesis. Recently, it was shown that genetically modified dense bodies containing

foreign proteins could be generated (Pepperl-Klindworth *et al.*, 2002). In light of this, it may be possible to produce dense body-based vaccines that are modified to improve their HCMV immunogenicity through the inclusion or enhanced expression of specific antigens. Dense body technology therefore, represents another innovative approach to HCMV vaccine development that deserves continued exploration.

Challenges for HCMV vaccine development

Species-specificity of HCMV

HCMV has a very limited host range, and no practical animal model for HCMV infection has been identified (Mocarski and Courcelle, 2001). While the CMVs of certain other animal species are well studied and very useful for addressing many aspects of vaccine development, they are genetically distinct from HCMV and may not behave identically during natural infection. Thus, for any new HCMV vaccine candidate initial safety and efficacy data can be derived from animal studies using analogous vaccines made from animal CMVs. However, this data will need to be interpreted cautiously, and valid safety and efficacy data will require human trials. Moreover, the species specificity of HCMV is an impediment for testing live, presumably attenuated vaccine candidates. Without the ability to assess HCMV virulence in an animal model, attenuation can only be confirmed through human trials. While this was accomplished for the Towne/Toledo chimeric vaccine candidates described above, it undoubtedly complicates vaccine development efforts.

Efficacy testing

The primary goal of HCMV vaccination is the prevention of congenital disease. However, given that the incidence of HCMV disease is about 0.1% of all births, a prospective trial to assess vaccine efficacy against HCMV disease would require tens of thousands of participants and a lengthy follow-up period to ensure that late sequelae of CMV infection, such as hearing and intelligence deficits, are not missed. However, an efficacy trial to determine the ability of a vaccine to protect newborns from HCMV infection could be done with a manageable number of subjects. Assuming a fetal infection rate of 1%, vaccine efficacy of 80% and a 2-year follow-up period, such a trial would require fewer than 5000 participants (Plotkin, 1999). Moreover, surrogate endpoints for vaccine efficacy could be tested initially to exclude less promising vaccine candidates from future large trials. For example, the ability of a vaccine to prevent infection in seronegative mothers with children in daycare would require less than 200 participants, given the high incidence of HCMV infection in that population (Plotkin, 1999).

When to vaccinate

To prevent congenital disease, HCMV vaccination should be administered to women prior to their becoming pregnant, and it has been proposed that vaccination of girls between the ages of 11 and 13 would be appropriate (Plotkin, 1999). However, the persistence of the immune response would need to be determined before a final schedule could be recommended. If a vaccine induced long-lived immunity, early childhood vaccination should be considered. If, however, immunity is less durable, later vaccination or regular boosters may be appropriate.

Summary

At present, vaccination remains the best hope for preventing most cases of congenital HCMV disease. Several new HCMV vaccination strategies have emerged in the past decade that take advantage of our current understanding of the host immune response to HCMV infection. Moreover, some older approaches, such as live attenuated vaccines, are being readdressed using modern molecular genetic approaches to enhance protective efficacy. More than ever, the development of a safe and effective HCMV vaccine seems to be an achievable goal. The most important scientific challenge that remains is to refine our understanding of the immunology of HCMV disease protection in order to rationally design vaccines that are both safe and effective. In addition, agreement must be reached within the medical and scientific communities on the best approaches for efficacy testing of new HCMV vaccines before the large trials necessary for licensure can be conducted.

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Epstein–Barr virus vaccines

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Introduction

Primates and their γ-herpesviruses enjoy a largely peaceful coexistence where a balance of power has been reached over evolutionary time. Coevolution probably began before primate speciation and has allowed these viruses to develop sophisticated systems for the evasion of host immune responses. As a consequence, herpesvirus vaccines have been especially difficult to design because of viral latency, persistence, and immune modulation. Epstein–Barr virus (EBV) persists for the life of the individual in the face of a range of antibody responses, some of which are virus-neutralizing in vitro and a multitude of cell-mediated responses, including viral-specific CD8+ T-cells, CD4+ T-cells and NK cells. At least 95% of the adult population is infected with EBV and, for the vast majority, there are no clinical consequences whatsoever and an asymptomatic carrier state is maintained. It is not clear whether advantages are conferred to humans by lifelong EBV infection, but it is possible that some immunological effects, such as bias of the T-cell receptor repertoire are provided on a population-wide basis. Whether unselective mass vaccination of healthy individuals to prevent or modify EBV infection may cause more problems than it would solve must be considered.

M.A. Epstein first put forward ideas on the development of EBV vaccines in 1976. These original proposals were based on the notion that vaccination might prevent EBV infection and break the link in the complex chains of events that lead to EBV-associated disease. Since that time, a better understanding of EBV biology has led to the elaboration of more sophisticated vaccination strategies. Presently, it seems most unlikely that vaccination of any kind will achieve sterilizing immunity against herpesviruses. The murine γ-herpesvirus, MHV68, establishes the same steady-state levels of lytic and latent infection whatever the route of infection or dose. It may be that a single virus particle successfully infecting a single target cell will be enough to establish persistent infection in a susceptible subject (Tibbetts, 2003). The goal of EBV vaccination is the prevention of disease and not of infection. Vaccination that could modify infection, or at least the subsequent immunological status of the infected person with respect to EBV, may prevent or minimize disease. It should be noted that EBV will not have evolved to evade vaccine-induced immune responses where they are qualitatively and/or quantitatively different from naturally occurring immune responses. An important precedent for herpesvirus vaccination is the attenuated varicella zoster virus (VZV) Oka strain vaccine that may not prevent infection but is able to prevent disease. More recently, the concept of therapeutic vaccination to treat EBV-associated tumors themselves has begun to emerge (Khanna *et al.*, 2001; Ong *et al.*, 2003; Khanna *et al.*, 2005).

An understanding of the life cycle and cellular habitats of EBV should be an essential prerequisite in the rational design of EBV vaccines. Unfortunately, the biology of EBV in vivo remains poorly understood and the various approaches to EBV vaccine design discussed below are, of necessity, based on a number of unproven assumptions. EBV is an orally transmitted infection and is able to infect B-cells travelling in the circulation in the oropharynx or resident in lymphoid tissue in this region. The point of infection is presumed to be the oropharyngeal epithelium but the identity of the primary target cell is not clear. It has not been possible to convincingly demonstrate the presence of EBV in oropharyngeal epithelial cells in vivo. Nevertheless, it has been shown recently, using polarized tongue and orophayngeal epithelial cells in vitro, that EBV-infected donor cells in saliva are very efficient at infecting recipient epithelial cells at their apical surface by cell–cell contact. However, these same epithelial cells are refractory to

infection with free virus at their apical surface. It is also shown that neighboring epithelial cells are infected by cell–cell transmission and free virus is produced at both the apical and basolateral epithelial surfaces (Tugizov *et al.*, 2003). Presumably, it is the latter cell-free virus that subsequently infects B-cells circulating within the oropharyngeal epithelium and oropharyngeal lymphoid tissues.

EBV infects and transforms B-cells in vitro and six EBV nuclear antigens, EBNAs 1 to 6 and two viral latent membrane-proteins, LMP1 and 2, expressed in the transformed B-cells are responsible for their changed growth and phenotype. These latency antigens potentially offer a range of targets for vaccine-induced cell-mediated immune responses. Healthy seropositive individuals carry CD8+ T-cells that are specific for epitopes in the latent antigens, in particular EBNA3, 4 and 6. However, EBV gene expression in infected B-cells in vivo is quite different and the growth-transformed phenotype has only been detected in the B-cell follicles of tonsils while in peripheral blood, EBV is detected only in a very small number of resting memory B-cells when EBV gene expression is restricted to LMP2 or does not occur at all (Thorley-Lawson, 2001).

A normal healthy immune response appears to be essential in maintaining the asymptomatic carrier-state as demonstrated by the occurrence of post-transplant lymphoproliferative disease (PTLD) in immunosuppressed organ transplant recipients and Non-Hodgkin's B-cell lymphomas in AIDS patients whose immune systems are seriously impaired. This strongly indicates that some aspects of EBV infection are under immune control and that vaccine-induced immune responses may be able to regulate primary infection and/or modify subsequent persistent infection. Most primary infections occur in the first few years in life and any prophylactic vaccine to control EBV diseases must allow for this. It would be a daunting task to deliver an EBV vaccine to large populations in Africa and China where primary infection occurs soon after birth.

Each EBV-associated disease probably arises for a complex set of different reasons and each may require a different vaccination strategy. Present approaches can be divided into those that seek to prevent or modify infection and those that might be used therapeutically to direct existing or de novo immune responses against the EBV-associated tumors. The therapeutic approach may prove to be particularly difficult, as, despite extensive efforts over a number of years, no tumors to date have consistently been controlled in humans by vaccine-induced immune responses. Modification of EBV infection may yet prove to be the correct approach and an interesting parallel may be drawn with a recent trial of a human papillomavirus (HPV) virus-like particle vaccine. In a trial involving 2392 young women, no cases of HPV 16 infection or cervical intraepithelial neoplasia were detected in vaccinated women in contrast to the control group (Koutsky *et al.*, 2002). Immunotherapeutic approaches to the treatment of cervical carcinoma have so far been unsuccessful. The logistics and costs associated with mass HPV vaccination of populations at risk will probably exceed those likely to be incurred with a prophylactic EBV vaccine and would at least set an important precedent if adopted as a public health measure.

Infection by more than one strain of EBV in both healthy and immunocompromised individuals appears to be unexceptional (Walling *et al.*, 2003). This raises difficult questions about the induction of immunity by EBV infection itself and about what vaccine-induced immune responses could realistically be expected to achieve. Infection by more than one strain simultaneously from one donor would not rule out the presence of subsequent virus-induced protective immune responses. However, if a second strain were able to infect an individual following an earlier infection with another strain, then it would suggest that the second EBV strain is able to evade the broad spectrum of humoral and cellular immune responses induced by the first EBV infection. A precedent for this situation has been found earlier with cytomegalovirus (Bale *et al.*, 1996). Another consequence of the existence of several strains of EBV is that strain differences will have to be incorporated into any prophylactic or therapeutic vaccine formulations that contain elements that differ between strains.

Since EBV infection is almost completely non-permissive in vitro, it is still not technically feasible to produce EBV itself on a large enough scale to support even a small vaccine trial where killed or attenuated forms could be used. Moreover, since EBV has been formally classified as a Grade I carcinogen (Ablashi *et al.*, 1997), and a number of its genes can independently transform certain cell types, the use of killed, attenuated or recombinant EBV vaccines can probably be ruled out for the time being. The possible composition of an EBV vaccine must be restricted to viral components that are non-transforming, however, this need not exclude non-transforming derivatives of viral transforming gene products such as synthetic peptides.

EBV causes infectious mononucleosis (IM), post-transplant lymphoproliferative disease (PTLD) and is associated with undifferentiated nasopharyngeal carcinoma (NPC), certain types of Hodgkin's lymphoma (HL), certain T-cell lymphomas, a subset of gastric carcinomas, and endemic Burkitt's lymphoma (BL). It is conceivable that a prophylactic vaccine or a postinfection vaccine that could modify but not prevent EBV infection could reduce the incidence of all diseases associated with EBV. The fact that increased antibody levels against virus capsid antigen

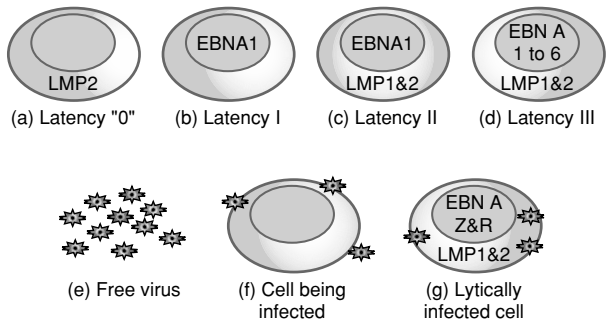

Fig. 72.1. Potential targets for EBV vaccine-induced immune responses.

In addition to the virus itself there are at least six different potential cellular targets for vaccine-induced immune responses. (**a**) Resting memory B-cells in which only LMP2 transcripts have been detected (Latence 0). These cells are few in number, there being only ~1 per 10^6 B-cells in the peripheral circulation, and are probably immunologically invisible; (**b**) BL cells are the only known example where EBNA1 is the sole EBV product and is usually referred to as Latency I. EBNA1 is not presented in an MHC class I context but is probably presented in a MHC class II context to CD4+ T-cell; (**c**) Infected B-cells in the default program where EBNA1, LMP1 and LMP2 are expressed. It has been hypothesized that this program allows for the differentiation of infected B-lymphoblasts into memory cells and provides signals necessary to maintain the infected blast cell against a background of immunological signals that would normally promote either proliferation or apoptosis. This program is often referred to as Latency II and the same or similar EBV latency gene expression profile is found in NPC and HD tumor cells; (**d**) All EBV latency genes are expressed and the cell is activated to become a proliferating lymphoblast and is referred to as being in the growth program or Latency III. This latency type is found in PTLD and some BL and could represent a good target for vaccine-induced immune responses through the EBNA3 family of products. (**e**) Free virus found in the oropharynx and at the basolateral face of the oropharyngeal epithelium may be subject to control by virus-neutralizing antibodies; (**f**) B-cells in the process of being infected may be the subject of antibody and cell-mediated control; (**g**) A small minority of infected B-cells will enter the lytic cycle and are potential targets for both cell-mediated and humoral responses.

(VCA) are prognostic for both BL and NPC suggests that immune intervention prior to the onset of disease could be beneficial. The success of such approaches will depend on whether the apparent reactivation of EBV is associated with the cause of NPC or is simply a consequence of tumor development.

Figure 72.1 illustrates the possible targets for vaccine-induced immune responses. These include free virus itself that might be susceptible to inactivation by vaccine-induced circulating or mucosal neutralizing antibodies. Naturally occurring virus-neutralizing antibodies are generated mainly against the major envelope glycoprotein, gp350 and much early work in EBV vaccine development focused on producing recombinant forms of this molecule (Morgan, 1992). Gp350 is also a target for cell-mediated responses (Khanna *et al.*, 1999a,b,c; Wilson *et al.*, 1999; Adhikary *et al.*, 2006). Other lytic cycle products such as gp85, gp42, the gB homologue and the products of the BZLF1, BMRF1, BDLF3 and BILF2 open reading frames have not been investigated in the context of vaccine development. CD8+ T-cell responses against BZLF1 predominate in healthy seropositives and vaccine-induced immune responses against BZLF1 in EBV seronegatives might have a role to play. It is not known how many EBV-infected B-cells enter the lytic cycle or the location of the cells that do. Very few B-cells infected and transformed with EBV in vitro enter the lytic cycle, being less than 5%. Since free virus is shed in the oropharynx, it is assumed that epithelial cells and/or B-cells in this region are responsible and might therefore be regulated by immune responses. It must be assumed for the time being that vaccine-induced immune responses against lytic cycle products would act on these cells and/or the virus they produce. The increased oral shedding of EBV in immunosuppressed patients is consistent with this view (Preiksaitis *et al.*, 1992). Current strategies to control EBV disease by vaccination are described below, while adoptive T-cell immunotherapy for these diseases is described elsewhere in this volume.

Vaccines to prevent infectious mononucleosis

In the wealthier Western societies primary EBV infection is often delayed until adolescence whereupon it gives rise to IM in 30% and 50% of individuals. It is still an open question as to why the remaining 50% to 70% of individuals become infected without symptoms or disease. However, it has been found that asymptomatic individuals display broad expansions of their TcR repertoire, while acute IM donors show oligoclonal expansion of TcR families (Silins *et al.*, 2001). Perhaps of even greater interest is the small minority who apparently never become infected. A higher production of IFNα and IL-6 and a greater number of monocytes were detected in cultures of peripheral blood lymphocytes from EBV-seronegative adults (Jabs *et al.*, 1996). The genetic and immunological differences between EBV seropositives and seronegatives remains obscure but would be informative as far as vaccine design is concerned. Since these immunological parameters are unknown, a vaccine cannot be designed on an entirely rational basis. IM almost always

resolves itself over a relatively short time and is only very rarely fatal. The question arises as to whether large-scale vaccination of otherwise healthy children is justified when set against the actual risks of illness and its socio-economic consequences.

Which stages in the process and maintenance of EBV infection are susceptible to control by vaccination? What target antigens need to be recognized by vaccine-induced immune responses to prevent IM? The differentiation pathways of EBV-infected cells, the types of B-cell that are infected and the EBV proteins expressed in vivo have been tentatively identified and include a memory B-cell that may only express LMP2, a proliferating and activated B-cell expressing the full panel of EBV latent genes in the growth program or Latency III, and B-cells in Latency II which may subsequently generate the memory reservoir B-cells (Thorley-Lawson, 2001). Cells in Latency III have only been found in the germinal center of lymphoid follicles and these are regions in which cytotoxic T-cells (CTL) are poorly represented probably because EBV-specific CD8 T-cells lack homing receptors for lymphoid infection sites (Chen *et al.*, 2001). CD4+ T-cells are detectable at low frequency within B-cell follicles and may, therefore, interact directly with EBV-infected B-cells at this site. It is possible that CD4+ T-cells primed by gp350 or latency antigen vaccination would become reactivated on viral challenge (Adhikary *et al.*, 2006). Such cells could influence the course of IM by inducing apoptosis of EBV-infected B-cells within infected lymph nodes and by down regulating the large monoclonal or oligoclonal populations of CD8+ T-cells that account for much of the lymphocytosis that is symptomatic of IM.

Prophylactic EBV vaccine development has focused on the gp350 and EBNA3 antigens. Systemic virus neutralizing antibodies can be easily induced by vaccination with gp350 and an appropriate adjuvant and, while epithelial cell infection may be unaffected, it is conceivable that free virus liberated on the basolateral surface of infected oropharyngeal epithelium could be neutralized and minimize transmission to circulating naïve B-cells. While infection itself will not be prevented, the effective virus dose at the B-cell level would be greatly diminished. The induction of mucosal immune responses in the orapharynx may prove to be more effective and the oral/nasal administration of gp350 in conjunction with mucosal adjuvants such as Iscoms (Wilson *et al.*, 1999), cholera toxin B-subunit or the *E.coli* heat labile enterotoxin B-subunit, EtxB (Williams *et al.*, 1999), should be investigated. However, there is some evidence that mucosal IgA specific for gp350 actually enhances infection of certain epithelial cells in vitro (Gan *et al.*, 1997).

The strategy would be to prevent or modify primary EBV infection by vaccination of children before primary infection. The disease itself is caused by excessive CD8+ T-cell responses to EBV infection, and in particular, to EBV lytic antigens. Any vaccine that could allow a more rigorous control of the primary infection phase may therefore prevent the disease or reduce its severity. Other explanations for the pathogenesis of IM, unconnected to the dose of virus at primary infection, are possible and include differences in NK and CD4+ T-cell responses (Wilson & Morgan, 2002), expansion of a CD28 subset of CD4+ T-cells (Uda *et al.*, 2002) and autoimmune responses (McClain *et al.*, 2003). Until these issues are better understood, it will be difficult to take account of them.

Gp350

The major EBV envelope glycoprotein, gp350, binds to the CR2 complement receptor on B-cells and is consistent with it being a target for neutralizing antibodies. Following attachment the virus infects the target cell through envelope fusion events involving other EBV envelope glycoproteins gp85, gp42 and gp25 (Borza & Hutt-Fletcher, 2002). More recent work has shown that gp350 is not an absolute requirement for EBV infection to take place. A recombinant EBV in which the gp350 gene had been deleted was able infect a range of B-cell lines and epithelial cells, albeit at a lower efficiency (Janz *et al.*, 2000). The early observation that serum EBV neutralizing antibodies largely recognized the major viral envelope glycoprotein, gp350 set the scene for subsequent work over a number of years involving a New World primate, the cottontop tamarin (*Saguinus oedipus Oedipus)*. Oligoclonal B-cell lymphomas closely resembling those seen in post-transplant lymphoproliferative disease (PTLD) can be routinely induced in these animals by injection of EBV. The characterization and purification of tractable quantities of the gp350 viral envelope glycoprotein from both natural sources and by recombinant DNA methods was carried out. Recombinant gp350 in combination with adjuvants or when expressed in vaccinia or adenovirus vectors, induced protective immunity in cottontop tamarins susceptible to EBV-induced B-cell lymphoma. Protective immunity was not dependent on the induction of gp350-specific neutralizing antibodies but was achieved through cell-mediated immune responses (Morgan, 1992). Gp350 vaccine formulations have since been shown to induce CTL responses as well as neutralizing antibody (Khanna *et al.*, 1999c, Wilson *et al.*, 1996). The mechanism of protection in this animal model is unknown. Did the protective responses induced in the tamarin act on the virus or on tumor cells? Since the tumor cells have

a Latency III phenotype and do not express gp350, the induced immune responses were probably able to reduce the effective virus dose on challenge since the induction of tumors by EBV is dose dependent. Derivatives of the gp350 vaccines described above (Jackman *et al.*, 1999) have been evaluated in human trials (Denis, 2005). The results of these trials strongly indicate that gp350 vaccination of seronegative young adults prevents IM but does not prevent EBV infection. Surprisingly, little attention has been placed on developing DNA vaccines coding for gp350. Immunization of mice with such a vector gave rise to antibodies to gp350, antibody-dependent cellular cytotoxicity (ADCC) and gp350-specific CTLs (Jung *et al.*, 2001).

The cottontop tamarin has a number of shortcomings as a model of EBV infection and disease. This species is not infected by the oral route and does not sustain a persistent infection. A further complication is that the tamarin has an unusually restricted histocompatibility complex polymorphism and only expresses the alleles G, F. and E, associated primarily with NK cell function (Cadavid *et al.*, 1999). Moreover, contrary to earlier beliefs, tamarins and marmosets have been found to carry their own resident γ-herpesviruses (de Thoisy *et al.*, 2003).

A recombinant derivative of the Chinese vaccinia Tien Tan strain expressing gp350 was used to vaccinate a small group of both seronegative and seropositive children in Southern China. Antibody levels to gp350 were raised in seropositive subjects and were induced in those who were seronegative at the beginning of the trial. Six out of nine vaccinated children who were seronegative for EBV at the time of vaccination remained seronegative for at least three years after vaccination (Gu *et al.*, 1995). The particular vaccinia recombinant used in this trial would not be currently acceptable for large-scale use on safety grounds. The Modified Vaccinia Ankara (MVA) strain would provide an acceptable alternative vector (Stittelaar *et al.*, 2001).

Some development work has been carried out on the generation of a recombinant varicella zoster virus (VZV) vaccine vector for the delivery of EBV genes and VZV recombinants were produced which are able to express EBV gp350 (Lowe *et al.*, 1987). It may be timely to explore this option further given the success of the Oka VZV vaccine strain and its incorporation into national vaccine programs in the USA, Japan and elsewhere.

A murine γ-herpesvirus (MHV 68) is increasingly being used to model EBV biology and this virus induces a mononucleosis-like syndrome in mice (Blackman *et al.*, 2000). Vaccination studies have been carried out using this model and the MHV 68 major envelope glycoprotein gp150, an analogue of EBV gp350 (Stewart *et al.*, 1996) was incorporated into a recombinant vaccinia virus vector and used to vaccinate mice prior to intranasal challenge with MHV 68. Virus-neutralizing antibodies were induced and the mononucleosis-like syndrome normally caused by MHV 68 was almost completely eliminated. MHV 68 latency was established in the vaccinated mice nevertheless (Stewart *et al.*, 1999). These results are a cause for some optimism in that appropriately administered EBV gp350 could prevent IM even if the establishment of latent, persistent EBV infection was unaffected.

EBNA3

Another approach using synthetic peptides based on the EBNA3 latent antigen, to induce cell-mediated immune responses, was developed in parallel and has also been the subject of small-scale human trials (Khanna *et al.*, 1999a,b,c). This approach utilizes latent antigen epitopes restricted through common MHC class I alleles to induce CD8+ T-cell responses in the vaccinee. Strong support for this approach came from the demonstration that autologous CD8+ T-cells against EBNA3, propagated ex vivo and introduced into immunosuppressed patients at high risk of PTLD, were able to prevent PTLD and in some cases cause PTLD to regress (Gottschalk *et al.*, 2002). In other words, CTLs specific for some EBV latent antigens can control the propagation of EBV-infected B-cell tumors *in vivo*. A Phase I human trial has been carried out to establish whether an EBNA 3 synthetic peptide, FLRGRAYGL, incorporated into a water-in-oil emulsion adjuvant, can be safely used to induce epitope-specific CTL responses (Moss *et al.*, 1998). Ultimately, a collection of epitopes will be used to span the majority of HLA types and encompass strain variation in target populations. Similar strategies may be adopted with peptide epitopes from lytic cycle antigens such as gp350 and BZLF1. Until it is known which T-cell specificities are important in protecting against IM, there may be a case for constructing an epitope vaccine using epitopes from both lytic and latent antigens. Vaccination with a latency antigen inserted into a DNA plasmid expression vector has been tested in the MHV 68 murine γ-herpesvirus model using the M2 latency-associated gene. M2 DNA vaccination had no effect on virus replication in the lung but did reduce the latently infected cell burden in the early, but not the later, stages of infection (Usherwood *et al.*, 2001).

One serious hurdle for peptide-based vaccines is the relatively large number of epitopes that would need to be incorporated into a single vaccine so that it could be delivered to a wide range of individuals with different HLA types. A vaccine formulation with so many components may face difficulties with regulatory approval. This problem can be overcome using polyepitope constructs (Thomson *et al.*, 1996). The polyepitope corresponds to the linking of minimalized CTL epitopes within a single coding sequence

as a "string of beads" and has been shown to be highly efficient in inducing protective CTL responses when delivered as part of a live viral vector or a recombinant DNA plasmid (Thomson *et al.*, 1998, 1996). Gp350 subunit vaccines avoid these problems since all possible epitopes are contained within the whole protein and sequence variation between different EBV isolates appears to be minimal. To what extent do CTLs reactivated from memory T-cells by autologous LCLs and assayed against peptide-coated targets, or targets infected with vaccinia recombinants, reflect *in vivo* CTL activities? The therapeutic effects on PTLD, of EBV-specific CTLs grown ex vivo, support the view that CTLs of one or other latent antigen are responsible for these effects (Sherritt *et al.*, 2003). Which cell types and which antigen specificities are responsible for the beneficial effects of infusion of *ex vivo* grown T-cells, is not yet clear.

Vaccines to prevent post-transplant lymphoproliferation disease

Approximately 10% of seronegative children receiving solid organ transplants develop PTLD during the first year after transplant. The five-year survival of patients who develop PTLD is poor being only 35% for renal transplant recipients and 26% for heart transplant recipients. The risk of developing significant morbidity or PTLD following primary EBV infection in non-immune transplant recipients is about 20 times greater than in seropositive transplant recipients. Pediatric transplant patients are much more likely to be seronegative than adult patients since EBV infection increases with increasing age. Primary EBV infection occurs in about 70% of all seronegative recipients during the first 6 months following transplantation. This is the period during which the most intensive immunosuppression occurs. Immunization of seronegative patients before transplantation provides a realistic opportunity to test whether the presence of antibody to EBV or residual EBV-specific T-cell responses still active during immunosuppression will protect against infection, spread or pathogenic effects of EBV following transplantation.

Therapeutic vaccines to prevent Hodgkin's disease and nasopharygeal carcinoma

Of the interventions designed to treat human malignancies, immunotherapy with CTL is increasingly being recognized as potentially the most efficient strategy with minimal side effects (Savoldo *et al.*, 2000; Bollard *et al.*, 2004; Straathof *et al.*, 2005). The key factors in the development of a CTL-based therapeutic strategy are the characterization of therapeutic immune correlates, and the delineation of the specific portions of the tumor-associated antigens that elicit these responses. $CD8^+$ CTLs are considered important for protection against various virus-associated malignancies and have emerged as the major element in the immune control of malignant cells (Khanna *et al.*, 1999b). Indeed, a number of studies have recently been published, which have shown that adoptive immunotherapy can be successfully used to reverse the outgrowth of polyclonal B-cell lymphomas in transplant patients (Khanna *et al.*, 1999a,b,c; Rooney *et al.*, 2002).

Approximately half of HD cases involve EBV-positive tumor cells while all cases of undifferentiated NPC are EBV positive. Prophylactic vaccination of seronegative children or seropositive adults could lead to the reduction or elimination in the incidence of HD and NPC. However, the logistics, potential costs, and timescale of such a program may be disproportionate when seen in the context of other health priorities such as hepatitis, cervical carcinoma, malaria, HIV and others.

Despite being quite different cell types and at different locations, HD and NPC tumor cells express the EBV latent antigens, EBNA1, LMP1 and LMP2 and these are potential targets for the immune system. It is, therefore, surprising that the immune system is not able to kill these cells and indicates that viral immune evasion mechanisms are operating. One aspect of this phenomenon is already partly understood in so far as EBNA1 includes a glycine-alanine repeat (GAr) domain that not only may block its proteasomal degradation but also inhibits EBNA1 mRNA translation in *cis* (Yin *et al.*, 2003). Whether EBNA1 is degraded and processed in an MHC class I pathway in epithelial cells seems to depend on the type of epithelial cell. When EBNA1 is artificially expressed in epithelial cells of squamous origin, it appears to both inhibit growth and be degraded in a way that renders the cells targets for HLA-matched EBNA1-specific CTLs. Neither of these phenomena were observed when EBNA1 was expressed in epithelial cells of glandular origin (Jones *et al.*, 2003). Despite the presence of significant numbers of EBNA1-specific T-cells generated by cross-priming (Blake *et al.*, 2000), NPC and HD cells are not effectively targeted. More recent studies have shown that epitopes from EBNA1 can be endogenously processed and presented on the cell surface. These epitopes are primarily derived from newly synthesized protein as defective ribosomal products (Tellam *et al.*, 2004). LMP1 and LMP2 are potential targets and CTLs specific for epitopes in these proteins are found in the circulation, albeit at relatively low levels and CTL responses to LMP epitopes are said to be subdominant in relation to CTL responses to epitopes in EBNA3 and in BZLF1. T-cell

responses and antibody responses against LMP1 have been difficult to detect in humans (Khanna *et al.*, 1998a; Meij *et al.*, 2002). Both LMP1 and LMP2 may interfere with their own MHC class I presentation and may be poor targets and/or weak inducers of CTLs (Dukers *et al.*, 2000; Ong *et al.*, 2003). LMP1 and LMP2 have a plethora of effects on the biochemistry of EBV-infected cells some of which could impact on antigenic processing and presentation as well as on cell growth and differentiation (Dawson *et al.*, 2003; Portis and Longnecker, 2003). Another important observation in regard to CTL recognition of LMP1 and LMP2 is that the majority of the T-cell epitopes from these proteins are processed through a TAP-independent pathway (Khanna *et al.*, 1996; Lee *et al.*, 1996). TAP-deficient LCLs expressing LMP proteins are more efficiently recognized by LMP-specific CTLs than TAP-positive LCLs. These observations suggest that in the absence of TAP, ER generated LMP epitopes are presented more efficiently. On the other hand, presentation of these epitopes may be significantly reduced if TAP is expressed, as the peptides originating from the cytoplasmic compartment compete with ER generated LMP epitopes. It is therefore tempting to speculate that LMP-positive NPC and HD cells may have evolved to maintain TAP expression which limits the presentation of CTL epitopes from LMP1 and LMP2 and thus allows these tumors to escape CTL recognition in vivo.

Broadly speaking, two types of approach to therapeutic vaccination to treat NPC, HD and BL can be adopted. First, specific enhancement of effector cell responses to EBV proteins expressed in these cancers and secondly, enhancement of the presentation of the antigens in question by the tumor cells themselves. CTLs specific for LMP2 are detected in the circulation of NPC patients but are not found in the tumor lymphocyte infiltrate. NPC and HD cells also appear to have a normal MHC class I presentation pathway, at least in terms of MHC class I and TAP expression (Khanna *et al.*, 1998b; Lee *et al.*, 1998). Since both these cancers express identical viral proteins, it is anticipated that common immunotherapeutic protocols may be developed. It is important to remember that even if antigen presentation is unimpeded and specific CTLs are present at the tumor site in sufficient numbers, numerous other mechanisms, not necessarily related to EBV products, may prevent tumors cells being removed by the immune system (Gandhi *et al.*, 2006). There are many elements of the CTL recognition and target cell destruction process that have not yet been evaluated for NPC and HD tumor cells. These could include the expression of coreceptors and adhesion molecules, production of cytokines that provide an inappropriate milieu for CTLs, production of T-cell inhibitory receptors such as Fas, altered proteasome function and other tumor cell-specific factors.

Recent studies on HL patients have indicated that regulatory T-cells and LAG-3 play a pivotal role in suppressing EBV-specific T cell immunity (Gandhi *et al.*, 2006.)

LMP polyepitope vaccines

Although adoptive transfer of EBV-specific T cells has recently been tested for the treatment of relapsed HD, only a limited long-term therapeutic benefit was observed (Roskrow *et al.*, 1998). One of the major limiting factors in the development of an efficient therapeutic strategy is the viral antigens expressed in these malignancies are not only poorly immunogenic but also in some cases have the potential to initiate an independent neoplastic process in normal cells. Thus a strategy which can overcome both these potential limitations is likely to provide a safe and long-term therapeutic benefit to cancer patients.

One such strategy involves the delivery of immunogenic determinants from LMP1 and LMP2 as a polyepitope vaccine. Indeed, initial studies from one of our laboratories have shown that multiple HLA class-I-restricted LMP1 CTL epitopes, when used as a polyepitope vaccine in a poxvirus vector, efficiently induced a strong CTL response and this response could reverse the outgrowth of LMP1-expressing tumors in HLA-A2 K^b mice (Duraiswamy *et al.*, 2003b, 2004). A polyepitope-based vaccine for HD and NPC has a number of advantages over the traditionally proposed vaccines, which are based on full-length LMP antigens. Previous studies from our laboratory have indicated that polyepitope proteins are extremely unstable within the cytoplasm and may be rapidly degraded as a result of their limited secondary and tertiary structure. In contrast, full-length LMP antigens are unlikely to be degraded rapidly and may initiate various intracellular signalling events leading to the development of secondary cancers at the site of injection. Another important advantage includes the ability of a polyepitope vaccine to induce long-term protective CTL responses against a large number of CTL epitopes using a relatively small construct without any obvious need for a cognate help. Finally, the polyepitope-based vaccine is also likely to overcome any potential problem with the prevalence of LMP1 genetic variants in different ethnic groups of the world (Duraiswamy *et al.*, 2003a).

Although the poxvirus polyepitope vaccine vector provides long-lived expression of encoded epitopes, there are concerns in terms of its safety profile with adverse side effects including postvaccine encephalitis when used in humans. Moreover, the poxvirus-based LMP1-polyepitope vaccine contained only HLA A2-restricted epitopes and the

HLA A2 allele is carried by only about 55% of the individuals in most populations. If a CTL-based therapy for NPC and HD is to be applicable to a significant number of patients, the target population must be presented through HLA alleles present at high frequency in the patient population. In this context, in addition to LMP1, LMP2-specific responses restricted through A11, A24 and B40 are of particular interest because these alleles are very common in the Southern Chinese population (A11, 56%; A24, 27%; B40, 28%), particularly where NPC is endemic. To overcome these potential limitations, a novel approach has been devised of activating LMP-specific CTL responses with a replication-incompetent adenovirus encoding multiple epitopes from LMP1 and LMP2 (Duraiswamy *et al.*, 2004). This replication-incompetent adenovirus vaccine contains both LMP1 and LMP2 epitopes restricted through HLA alleles common in different ethnic groups including NPC endemic regions (HLA A2, A11, A23, A24, B27, B40 and B57). It has been estimated that these optimally selected MHC class I-restricted epitopes would include more than 90% of the Asian, African and Caucasian populations. Attractive features of adenovirus-based vaccines are their well-characterized genetic arrangement and function, as well as their extensive and safe usage in North American army recruits without inducing adverse side effects (Imler, 1995). Adenovirus-based vectors are being increasingly recognized for high efficiency and low toxicity and have been used in multiple human gene therapy clinical trials and preclinical vaccine applications. These vectors are also increasingly being used for cancer immunotherapy (Kusumoto *et al.*, 2001). Two of the most promising recent reports were studies in non-human primate models of the Ebola virus and HIV (Shiver *et al.*, 2002; Sullivan *et al.*, 2000). In each study, an immunization regimen that included priming with plasmid DNA followed by boosting with adenovirus vector particles showed the induction of effective CTL responses when compared with the plasmid DNA alone (Sullivan *et al.*, 2000). This new polyepitope vaccine is based on an E1/E3-deleted recombinant adenovirus comprising a chimeric Ad5/F35 vector that has been engineered to substitute the shorter-shafted fiber protein from Ad35 strain. This expression system provides an advantage over previous Ad5 vectors with respect to efficiency of expression of recombinant protein in hematopoietic stem cells and dendritic cells (Yotnda *et al.*, 2001). Our studies with the Ad5F35 LMP polyepitope vaccine have shown that each of the epitopes in this vaccine is not only efficiently processed endogenously by the human cells but also recalls memory CTL responses specific for LMP antigens in healthy virus carriers and HD patients. Furthermore, the adenoviral polyepitope vaccine is capable of inducing a primary T-cell response, which was shown to be therapeutic in a tumour challenge system (Smith *et al.*, 2006).

Altered antigen processing of LMPs using bacterial toxins

Since NPC is an epithelial tumor at a mucosal surface, the question of whether mucosal vaccine adjuvants might have a role to play in their treatment has been considered. One such adjuvant that has reached an advanced stage of development is the cholera toxin-like *E. coli* heat labile enterotoxin B-subunit (EtxB) (Williams *et al.*, 1999). Bacterial protein toxins are molecules that combine unique cell binding with efficient cytosolic delivery properties. Toxoid derivatives of the adenylate cyclase toxin of *Bordetella pertussis*, pertussis toxin, anthrax toxin, and Shiga toxin B subunit have been investigated as potential vehicles for delivery of exogenous peptides or proteins into the MHC class I presentation pathway (de Haan and Hirst, 2002). Recent work has established that EtxB possesses important features that makes it uniquely placed to be used as a delivery vehicle for MHC class I-restricted T-cell responses (De Haan *et al.*, 2002).

LMP-1 and LMP-2 are colocalized within plasma membrane GM_1-rich lipid rafts in LCLs (Higuchi *et al.*, 2001). LMP1 can be ubiquitinated and degraded through a proteasome pathway (Aviel *et al.*, 2000) while the LMP2 intracellular N-terminal polypeptide binds Nedd4-like ubiquitin ligases and is also subject to proteasomal processing (Ikeda *et al.*, 2002). MHC class I-restricted antigen presentation of LMP2 is unusual as some, but not all, epitopes are presented independently of TAP (Khanna *et al.*, 1996; Lee *et al.*, 1996). It is unclear at present why LMPs are not always effectively presented to CTL by LCLs in the absence of peptide epitope pre-sensitization or LMP2 expression by recombinant vaccinia. However, it has been shown that the treatment of EBV-positive LCLs with EtxB results in colocalization, capping and internalisation of LMP1 and 2. EtxB itself binds the ganglioside, GM_1, at the plasma membrane, enters the cell by endocytosis, and then the trans-Golgi network or endoplasmic reticulum by retrograde trafficking. LCLs treated with EtxB show a greatly increased susceptibility to killing by LMP1 and LMP2-specific CTLs (Ong *et al.*, 2003). The mechanism by which EtxB causes this enhancement of antigen presentation by LCLs requires further investigation but these results indicate that EtxB interferes with the normal distribution and pathways of LMP turnover (Ong *et al.*, 2003). The possibility therefore exists that EtxB could serve both as a mucosal adjuvant in the conventional sense by enhancing mucosal immune responses in the nasopharynx and also by enhancing the

presentation of LMP1 and LMP2 in NPC tumor cells in vivo. Further work is needed to establish whether EtxB can enhance the CTL killing of EBV-infected epithelial cells expressing LMP1 and/or LMP2 as it does for LCLs. To this end, it has recently been shown in one of the authors' laboratories that EtxB enhances the killing by LMP2-specific CTLs of H103 oral epithelial carcinoma cells expressing LMP2 (O. Salim, A. D. Wilson and A. J. Morgan, unpublished data).

One explanation for the failure of the immune system to kill HD and NPC tumor cells is that expression levels of the main potential immunological target, LMP2, are too low. Indeed, the unequivocal detection of LMP2 protein as opposed to RNA transcript in NPC cells has yet to be achieved. An EBV-transformed LCL, stably transfected with a plasmid expressing LMP2A under the control of an ecdysone analogue Ponasterone A (No *et al.*, 1996), has been created in one of our laboratories. Following exposure to Ponasterone A, the level of LMP2A was increased ten fold and was localized in the plasma membrane. Increased LMP2A expression resulted in the up-regulation of LMP1 expression, and had a blocking effect on the EBV spontaneous lytic cycle by down-regulating the expression of both the BZLF1 and BRLF1 genes. In normal LCL, LMP2 is not efficiently processed or presented to CTLs by MHC class I (Dukers *et al.*, 2000; Ong *et al.*, 2003). The tenfold increase in LMP2A expression induced by Ponasterone A did not result in any increase in lysis by an MHC class I restricted LMP2A specific CTL line. Susceptibility to CTL lysis was enhanced by treatment with EtxB but the enhancement was only marginally higher in the Ponasterone A-induced targets compared to the controls with normal levels of LMP2 (G. Patsos, A.D. Wilson, and A.J. Morgan, unpublished data). These data suggest that LMP2 processing and presentation is impaired in some way and that access to different, more efficient presentation pathways, will render LMP2-expressing cells more susceptible to specific CTLs.

Therapeutic vaccines for Burkitt's lymphoma

Whether prophylactic vaccination with EBV gp350 or latent antigen polyepitope formulations would prevent or reduce the incidence of endemic BL remains an open question. Raised levels of antibody against VCA are prognostic for BL and it has been suggested that control of EBV replication by vaccination before primary infection or before ant-VCA levels rise may have a protective effect. The prospect of devising a therapeutic vaccine for BL is rather problematical because of the absence of MHC class I-mediated antigen targets. Endemic BL cells were thought to express EBNA1 only but it has recently been found that three out of fifteen EBV-positive endemic BLs tested also expressed a truncated EBNA LP, EBNA3a, 3b and 3c (Kelly *et al.*, 2002). Although CTLs specific for the EBNA3 family may be abundant in these patients, it has been argued that the absence of LMP1 expression in the tumor cells does not allow the up regulation of antigen processing machinery. LMP1 may upregulate antigen-presenting functions in B-cells through an NF-κB pathway (Pai *et al.*, 2002).

The restriction of EBV gene expression to EBNA1 and a consistent loss of antigen processing function through the MHC class I pathway in BL cells, severely restricts the potential use of antigen-specific immunotherapeutic strategies. However, recent studies have provided some promising alternative therapeutic strategies for these malignancies. One such strategy involves targeting the tumor cells through virus-specific CD4+ CTLs. Previous studies from one of our laboratories have shown that BL cell lines displaying antigen processing defects through the MHC class I pathway are efficiently recognized by EBV-specific CD4+ CTLs. Furthermore, these tumor cells also express normal levels of all the essential components involved in the processing of T-cell epitopes through the MHC class II pathway. The importance of these studies has been further strengthened by the observation that CD4+ EBNA1-specific CTLs from healthy virus carriers can efficiently recognize virus-infected normal B-cells as well as BL cells expressing EBNA1 only (Munz *et al.*, 2000; Paludan *et al.*, 2002). These observations demonstrate that it may be possible to target EBNA1 through the MHC class II pathway (Paludan *et al.*, 2005). It raises the possibility that a vaccine based on EBNA1 that induces a strong CD4 T cell response may provide therapeutic benefit to BL patients.

One attractive way to deliver EBNA1 through the MHC class II pathway is to enable this antigen to gain access to endosomal or lysosomal compartments. There are two major pathways by which antigens are targeted to these compartments. The traditional pathway involves the phagocytosis or endocytosis of exogenous antigens, followed by degradation by acid proteases in the endosomal or lysosomal compartments. On the other hand, MHC class II-restricted presentation of endogenously synthesized proteins mainly involves membrane antigens that are thought to enter the endosomal or lysosomal pathway by internalization from the cell surface. The lysosome-associated membrane protein (LAMP-1) and the invariant chain are transmembrane proteins, which are predominantly localized in the lysosomes and endosomes, respectively. The cytoplasmic domains of these proteins contain specific targeting or address signals that mediate their translocation to the specific compartments. Previous studies from one

of our laboratories have shown that these targeting signal sequences can be utilized to direct multiple MHC class II-restricted CTL epitopes into the endosomal and lysosomal compartments (Thomson *et al.*, 1998). This approach not only preferentially translocates the polyepitope protein to these compartments but also enhances endogenous presentation of CTL epitopes. Furthermore, this strategy was successfully used to activate a virus-specific memory CTL response from peripheral blood lymphocytes. Endosome/lysosome-targeted EBNA1 is currently being tested as a therapeutic vaccine in a non-immunogenic murine B cell tumor model in which the tumor cells express EBNA1. If this vaccine strategy is successful in reversing the outgrowth of these tumor cells, it is possible that an EBNA1 vaccine may not only be applicable to BL but also against other EBV-associated malignancies such as HD and NPC.

Another interesting strategy involves treatment of the BL cells with soluble CD40 ligand (CD40L) (Khanna *et al.*, 1997). This treatment is highly effective in reversing the down-regulated expression of antigen processing genes involved in MHC class I-restricted presentation. Moreover, CD40L-treated BL cells regain susceptibility to EBV-specific CTL-mediated lysis. These data suggest that direct infusion of soluble CD40L at tumor sites or cytokine-mediated induction of CD40 ligand on bystander lymphocytes should be considered as an alternative approach to restoring immunogenicity of malignant cells. Taken together, these preclinical studies provide an important platform for the development of a therapeutic strategy for BL based on a combination of EBNA1 vaccination and CD40L injection.

Recently, a novel approach to override the GAr-mediated proteosomal block on EBNA1 by specifically targeting this antigen for rapid degradation by a process of cotranslational ubiquitination combined with N-end rule targeting, has been explored (Tellam *et al.*, 2001). These studies showed that enhanced intracellular degradation of EBNA1 was coincident with an induction of a very strong EBNA1-specific CTL response, and restored the endogenous processing of MHC class I-restricted CTL epitopes within EBNA1 for immune recognition by EBV-specific CTLs. It has also been shown recently that EBNA1 can be degraded in some epithelial cells of squamous origin and these cells are subsequently susceptible to MHC class I-restricted, EBNA1-specific CTLs (Jones *et al.*, 2003). These observations raise the possibility of developing therapeutic strategies to modulate the stability of EBNA1 in normal and malignant cells. These schemes may involve treatment of virus-infected cells with synthetic or biological mediators capable of enhancing stable ubiquitination and rapid intracellular degradation of EBNA1 in vivo. Because the substrate specificity of the ubiquitin-proteasome pathway is conferred by the E3 ubiquitin-protein ligases, then one approach may involve manipulation of the ubiquitin-dependent proteolytic machinery by targeting specific E3 ubiquitin-protein ligases to direct the degradation of otherwise stable cellular proteins, such as EBNA1. Potentially, this engineered proteolysis system could be utilized as a therapeutic method to counteract the proteasomal block conferred on EBNA1 through the *cis*-acting inhibitory GAr domain.

The MHV 68 model of γ-herpesvirus infection has indicted a role for CD4+ T-cells in both the control of infection (Hogan *et al.*, 2001) and in the control of tumor cells expressing MHV 68 antigens. When MHV 68-infected S11 cells were injected subcutaneously into nude mice, adoptively transferred lymphocytes caused the regression of S11 tumors and CD4+ T-cells were most effective in preventing tumor formation. CD4+ T-cells were also present in the regressing tumors (Robertson *et al.*, 2001).

Conclusions

The limitations imposed on EBV vaccine design include (1) the behavior of EBV in vivo is poorly understood such that the anatomical location of infected cells, the site at which they become infected, and the site at which new virus is produced, are not known with confidence; (2) the correlates of immune protection against, or modification of, in vivo EBV infection are unknown; (3) killed or attenuated variants of EBV cannot be used as vaccines because of the oncogenic potential of the virus and a number of its components; (4) the virus infection persists for life in the face of humoral and cell mediated immune responses; (5) although numerically very significant, only a very small proportion of the infected population will develop disease. Despite the fact that recent human trials have indicated that gp350 vaccination can prevent IM, but not EBV infection, in seronegative young adults (Denis, 2005), the immunological basis for this is remains unclear. Until further human trials have taken place it is unlikely that any correlates of protective immunity against either infection or disease will become known with only limited information coming from animal models of related viruses. It is impossible to say at this stage what effects gp350 vaccine-induced mucosal or systemic immune responses might have on primary EBV infection or existing EBV infection. Once latency has been established, will immune responses in the vaccinee be more effective in preventing EBV disease than in a normal unvaccinated seropositive? Large scale and unselective prophylactic mass vaccination are presently impractical given current public health priorities

and may even be unwise. Unless a clear benefit of such vaccination in a sizeable population can be demonstrated, such as in young seronegative adults in Western countries, resources will inevitably be focussed on therapeutic strategies for those who have already developed disease. Our increased knowledge of the antigen presentation and processing pathways of the main EBV-associated tumours, NPC and HD, has given rise to rational and novel immunotherapeutic strategies based on the enhancement of either LMP-specific CTLs or enhancement of LMP antigen presentation in the tumours themselves. Until recently, there were no immunotherapeutic strategies for treating endemic BL but the induction of appropriate CD4+ T-cells by vaccination may offer a way forward. The conducting of properly controlled human trials to evaluate the options set out above is clearly the first priority and little further progress can be expected until they take place.

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73

DNA vaccines for human herpesviruses

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General design of DNA vaccines

DNA vaccines are circular, double-stranded plasmid DNA (pDNA) molecules, which are capable of initiating the expression of protein antigens of interest when introduced into cells. For this purpose, the pDNA contains an eukaryotic expression cassette consisting of a transcriptional promoter, a protein coding sequence derived from the target antigen gene, and a transcriptional terminator (Fig. 73.1). Although many different promoters have been investigated, none have been shown to be clearly superior to the constitutive CMV IE promoter. DNA vaccines can consist of single genes on one plasmid, multiple genes on one plasmid, multiple plasmids, or a combination of the above. In biscistronic or tricistronic constructs, internal ribosomal entry sites (IRES) or equivalent sequences, dual or triple promoters, or cleavable linkage regions in fusion proteins can be used for expression of multiple genes. Upon transfer into cells, the pDNA enters the nucleus and transcribes a messenger RNA (mRNA) encoding the antigen of interest. The antigen can be identical to the wild-type protein of the pathogen, or can be genetically modified to improve immunogenicity and/or reduce toxicity to the host. The pDNA may also contain an antibiotic resistance gene and a bacterial origin of replication for growth and propagation in *E. coli*. Constructs using selection elements for bacterial replication other than antibiotic elements have also been utilized.

For vaccination, the purified pDNA is reconstituted in aqueous vehicles, or formulated and injected. A variety of delivery routes have been used, including intramuscular, intradermal, subcutaneous, transcutaneous, and mucosal (Ulmer, 2001). In the mouse, the route of delivery (e.g., intradermal versus intramuscular) may affect the antibody levels as well as the production of antigen specific Th1-like or Th2-like cytokines (Feltquate *et al.*, 1997). DNA vaccines, in which *de novo* production of the relevant immunogen is achieved without the use of live agents, combine some of the positive aspects of immune stimulation inherent in live-attenuated vaccines, such as enhanced cellular immune responses, with the safety and defined antigenicity of recombinant subunit vaccines. Furthermore, pDNAs expressing different antigens can easily be co-administered in a single formulation, enabling multivalent vaccines to be designed and tested with relative ease.

Mechanism of action

To develop rational approaches for enhancing DNA vaccine potency, an understanding of the mechanism of action of DNA vaccines is essential. The mechanism by which immune responses are initiated after intramuscular DNA vaccination has been a subject of debate for many years. Early discussions centered around three possible mechanisms: direct transfection of APCs, transfer of antigen from transfected muscles to APCs, or direct T cell priming by transfected myocytes (Padoll and Beckerleg, 1995). Data from experiments in which transfected myoblasts from H-2^k mice were transplanted into naïve H-2^d $\times$ H-2^k mice indicated that transgene encoded antigen could be transferred from the transfected myoblasts to host APC for generation of an antigen specific immune response (Ulmer *et al.*, 1996). Additional reports from experiments in which chimeric mice were used to characterize the role of bone marrow-derived APCs in priming immune responses after intramuscular DNA vaccination supported the hypothesis that antigen produced by muscle cells is transferred to APCs for initiation of antigen specific immune responses (Doe *et al.*, 1996; Corr *et al.*, 1996). While these experiments ruled

out a major role for direct T-cell priming by muscle cells, and indicated that antigen could be transferred to bone marrow-derived APC for T-cell priming, other experiments suggested that muscle expression is not critical for the generation of antigen specific immune responses after intramuscular DNA vaccination. Experiments in which injected muscles were removed within 1 to 10 minutes after injection, without diminishing the resulting B- and T-cell immune responses, argued for direct APC transfection (Torres *et al.*, 1997). A more recent study suggested that both mechanisms, direct transfection of bone marrow derived APC and cross-priming with antigen produced by muscle cells, are involved, and that cross-priming by antigen transfer is the predominant mechanism (Corr *et al.*, 1999). Increased local inflammatory responses with a resultant increase in APC migration to the muscle may play as great a role in cross-priming as the actual expression of gene product in the muscle. Furthermore, much of the pDNA injected into the muscle may be transported directly to the lymph nodes via the lymphatics, and thus the studies of Torres *et al.* may involve mechanisms other than direct APC transfection (Mena *et al.*, 2001).

The role of dermal cell expression of DNA vaccine-encoded antigens after intradermal or gene gun vaccination is still unclear. Direct transfection of APCs upon dermal injections of DNA vaccines may play a more extensive role in the induction of immune responses to DNA vaccination than the cross-priming that occurs in muscle cells. APCs such as Langerhans cells and macrophages are more numerous in the dermis and direct transfection of these cells has been shown after intradermal DNA vaccination and DNA vaccination by gene gun (Raz *et al.*, 1994). However, ablation of transfected dermal tissue within 24 hours of gene gun inoculation greatly diminished the antigen specific antibody response and precluded the generation of antigen specific CTL, suggesting a role for transfected dermal cells, other than migrating APCs, in the generation of an immune response after gene gun vaccination.

It is now generally believed that multiple mechanisms: cross-priming of APCs in the muscle or skin, direct transfection of APCs transiting the tissues, and uptake of pDNA by cells in the lymphatics or lymph nodes, are involved in immune response induction after DNA vaccination. However, in all of these cases, there is reason to believe that improved transfection of APCs, facilitated by the appropriate signals to allow for increased antigen or pDNA uptake, maturation, and migration to the local lymph tissues, will be critical in improving responses to DNA vaccines.

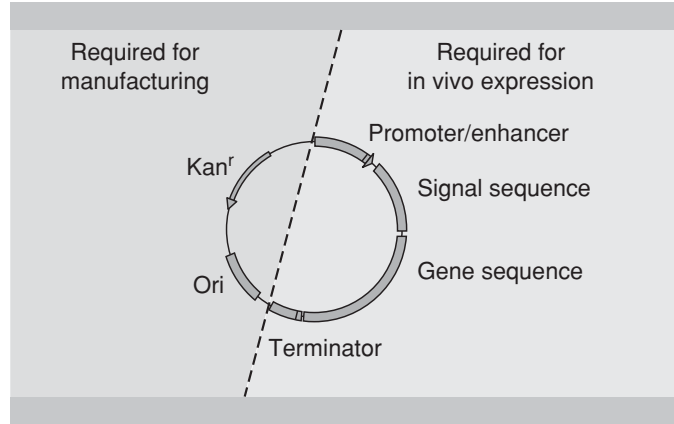

Fig. 73.1. Basic technology: plasmid DNA.

DNA vaccines: advantages and disadvantages

DNA vaccines have many advantages over other platforms, with the result that these products are used extensively as vaccine research tools. Many of these same advantages may facilitate development of therapeutic or prophylactic vaccination for herpesvirus infections (Table 73.1). In general, because gene constructs are easily modified, a very large number of differing constructs and combinations of immunogen genes can be rapidly developed and tested, which is not feasible with recombinant proteins. Injection of DNA can be readily used to investigate the function or protective effect of specific genes, combinations of genes, or effect of gene modifications. The combination of predictive algorithms for MHC Class I and II epitopes, combined with DNA vaccination in transgenic mice, has proven to be a powerful approach in antigen discovery for vaccination strategies targeting T-cell immunity.

In order to increase safety, target immunogens can be genetically modified to specifically, stably and uniformly remove biologically active regions of the proteins. For example, kinase or transactivating activity of key herpesvirus proteins can be removed, and yet the genes that contain the key T-cell or B-cell epitopes, regions, or exons of the gene can be retained. The effect of using a secretory leader or removal of transmembrane and cytoplasmic sequences can also be readily evaluated. Furthermore, the effect of glycosylation sites, minor amino acid changes, or the effects of combination genes that may form neutralization complexes (such as the gH/gL/gO complex in CMV, gH/gL complex in HSV, or gE/gI complex in VZV) can be much more quickly investigated, than when using techniques that apply recombinant protein or viral vectors.

Table 73.1. DNA vaccination approaches for herpes viruses

Advantages	Disadvantages
• Manufacture process and analytical testing are the same across different constructs • Stability • Time to clinic • Safety • Ability to titrate immune response to humoral or T-cell arms based on formulations/adjuvants • Ease of multivalent approaches • Ability to modify or detoxify the genes of interest • Lack of prior immunity or immune responses to the plasmid backbone	• Suboptimal responses in human trials to date • Regulatory pathway influenced by gene therapy • Population fear of "genetic" immunization • Not yet manufactured to scale • Possible need for more than a single injection to induce responses in humans

Herpesvirus subgroup	Virus	Tumor association
alpha	HSV-1	none
	HSV-2	none
	VZV	none
beta	CMV	none
	HHV-6	none
	HHV-7	none
gamma	EBV	Burkitts lymphoma, Gastric carcinoma, Nasopharyngeal carcinoma, Hodgkin's disease, Post-transplant lymphoproliferative disease, T-cell lymphoma, Leiomyosarcoma
	HHV-8	Kaposi sarcoma of HIV positive and negative patients, body cavity lymphoma, Castleman's disease

In the past decade, improvements in both plasmid design and formulations have led to approximately a 5-log increase in expression in muscle of the reporter gene, luciferase. The last log of this increase has been achieved using electroporation, which is being studied in man (Fig. 73.2). However, it is important to realize that *in vitro* or *in vivo* expression of the gene construct does not strictly equate with immunologic response to the DNA vaccine.

For the administration of plasmid DNA, both in animals and humans, a range of suitable DNA formulations have been established that can enhance either humoral or cellular immune memory to provide appropriate immune

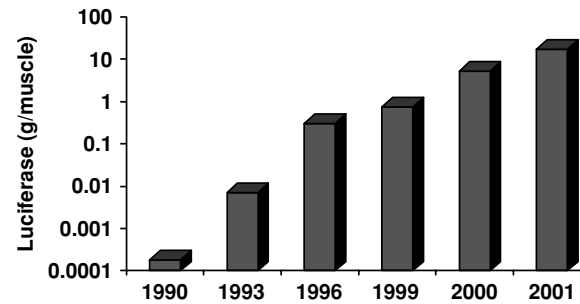

Fig. 73.2. Increase in the expression of the luciferase gene in vivo in muscle over the past decade (Vical in house data)

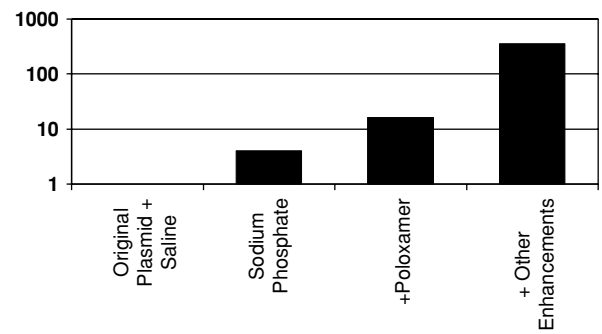

Fig. 73.3. Improvements in T-cell responses by formulation and delivery mechanism

responses for protection against the target pathogen. For example, the use of cationic lipids such as Vaxfectin drives a stronger humoral response than is achieved with unformulated pDNA, (Hartikka *et al.*, 2001), whereas formulation of DNA vaccines with poloxamers tends to enhance T cell responses but has less of an effect on B cell responses (Shiver *et al.*, 2002) (Fig. 73.3).

Thus, a single plasmid DNA vaccine can be used in animal models to define potential correlates of protection by simply changing the DNA vaccine formulation or route of administration. As an example, in the influenza murine model, the use of pDNA encoding NP is protective when given intramuscularly and generates CD8+ T-cell responses, (Ulmer *et al.*, 1993) but is less effective when given intradermally by the gene gun route which generated greater antibody responses (Robinson *et al.*, 1997).

Unlike genetic vaccines that use viral or bacterial vectors to deliver DNA encoded antigens, with DNA vaccines there are no problems with pre-existing or induced immunity to the DNA vaccines that could blunt or abrogate the desired immune response. Thus, repeated boosting over many years appears feasible, if necessary. In this regard, DNA vaccines may also be superior to the use of HSV or CMV as homologous or carrier vaccine vectors to induce

an immune response to a subdominant, but possibly more protective antigen.

A major advantage of DNA vaccines or DNA immunotherapeutics is that they are well tolerated and have been demonstrated to be safe in animals and in over 1000 humans. There has been persistent concern in the use of DNA vaccines with the potential for integration into the cellular DNA, but no published preclinical animal studies or human trials have demonstrated integration. In the preclinical studies published to date, the rate of integration has been shown to be at least 1000-fold lower than the risk posed by spontaneous mutations in the human genome (Martin *et al.*, 1999; Ledwith *et al.*, 2000).

DNA vaccines with many different antigen configurations can be produced with the same manufacturing platform, greatly simplifying the production process. Likewise, analytical testing of the plasmid varies little from one DNA vaccine to another, and downstream processing is relatively straightforward. Therefore, the time required to move from concept to animal testing and to the clinic is markedly shortened. Multiple plasmids can be processed in the same facility, and then combined in a final vaccine formulation. Properly formulated DNA vaccines can be stored in liquid form at refrigerated or room temperature for many years. Lastly, DNA vaccines do not require the handling of the pathogen or any mammalian cell substrates (with the associated risk of adventitious agents) at any point in the manufacturing process.

At present, the disadvantages of a DNA vaccine approach are similar to those of many emerging, disruptive technologies. The greatest criticism is the lack of substantive data in humans (see discussion below). The regulatory hurdles surrounding DNA have been originally placed nearly on level with those of gene therapy, and are only now beginning to be adjusted for the safety profile and knowledge of the technology that has emerged in the past decade. In addition, there will be a need to overcome the general fear in the public that this is not a "DNA altering" technology. Lastly, although DNA vaccines will eventually be quite inexpensive to produce, the technology is only now beginning to achieve the efficiencies of scale (such as in line fermentation, lysis, and column purification) that will drive down the costs of manufacture.

Standard DNA technology involves selecting individual genes and cloning them into a vector. A unique method of using DNA for herpesvirus vaccine studies is to inject the DNA as a provirus, which has been modified to remove only one or a few genes. This is now feasible with the advent of recombinant bacmid (bacterial artificial chromosome) technology, which allows for an infective proviral DNA construct to be made using *E. coli.* (Hahn *et al.*, 2003; Suter *et al.*, 1999). Such an approach has focused on removal of the immune evasion genes, to both decrease virulence and promote a greater immune response to the defective virus. Unfortunately, the bacmid can usually only be propagated as a single plasmid per single bacterium, and thus the yield from a fermentation is quite low. In general, the bacmid is then often propagated as a defective virus using a classic CMV-competent or trans-supplying cell line, and results in a replication defective virus for use in the vaccine trials. Although the injection of the bacmid into the host would likely result in some degree of defective virion production, the regulatory and safety hurdles surrounding such an approach would not be trivial.

Features of pDNA constructs especially conducive to herpesvirus vaccine development

As reviewed in previous chapters, there is minimal data that true sterilizing immunity to herpesvirus infections can be easily achieved through any present vaccine strategy. It may be a more realistic goal to limit the initial viral spread and thereby reduce the number of initially infected neurons (for HSV and VZV), or to limit the extent of reactivation of virus (EBV and CMV). Whether control of viral replication can best be achieved by use of a vaccine designed to induce antibody or T-cell-based immunity may depend on the clinical setting. As discussed below, prevention of CMV maternal-fetal transmission may be mostly antibody mediated, whereas prevention of CMV disease following infection in the hematopoietic cell transplant patient appears to be mostly T cell mediated. Nevertheless, most vaccine approaches to herpesvirus infections may require a greater degree of T-cell-mediated immunity than an approach to some other acute viral infections. The result of the HSV subunit trials has shown that adjuvants that bias toward a Th1 response may play a role in protective efficacy (Koelle and Corey, 2003).

DNA vaccines administered intramuscularly have been shown to induce balanced B- and T-cell responses, and will elicit CD8+ T-cell responses, which protein subunit vaccines, in general, cannot (Ulmer, 2001). Recent studies in macaques have shown that the T-cell responses induced by plasmid vaccination tend to be evenly balanced between $CD4^+$ and $CD8^+$ T-cells, whereas responses to adenovirus vectors are generally skewed toward $CD8^+$ T-cell responses (Casimiro *et al.*, 2003). A balanced T-cell response may be important in Herpesvirus infections, in which the role of $CD4^+$ T-cells as well as $CD8^+$ T-cells has been implicated in protection (Koelle and Corey, 2003).

In addition, as demonstrated in prime-boost experiments, DNA vaccines may be superior to other vaccine modalities in promoting long-term memory (McShane, 2002). This priming of memory responses may lead to a greater degree of recall responses, both T-cell and antibody, upon boosting by natural primary infection or viral reactivation. For example, monkeys that were immunized with a candidate authrax DNA vaccine were protected from a fatal challenge and manifested a rapid and rigorous recall response, despite a lack of protection neutralizing antibody at the time of challenge of viral data (Letvin *et al.*, 2006). It is not presently known whether this long-term T-cell memory response may be more critical in vaccine induced protection than the present focus on increasing the number of antigen specific effector cells quantified shortly after vaccination at the peak of the response.

DNA vaccines also have an advantage over the use of conventional live attenuated herpesvirus vaccines, such as the CMV Towne strain vaccine, in that they can be designed to encode relevant antigens without including the multiple immune evasion genes carried by human herpesviruses (Mocarski, 2002). In natural infection, many of these immune evasion gene products are designed to downregulate the T-cell response, and thus avoid recognition and lytic or non-lytic removal by $CD4^+$, $CD8^+$ or NK positive T-cells. Because immune evasion gene products may preclude responses to antigens produced during late infection, the antigens likely to be efficiently recognized by T-cells are those produced early in the infection process such as those encoded by immediate early genes. In addition, preformed structural proteins carried in by the virus may access the Class I pathway directly following infection of the cell or the Class II pathway as exogenous antigen (Tabi *et al.*, 2001; McLaughlin-Taylor *et al.*, 1994) and result in T-cell responses.

Additionally, the amount of early antigen proteins made by conventional live attenuated vaccines may be small as compared to the physical mass of structural proteins, and thus the immune response may be skewed in part by attenuated or vectored vaccines to these late gene products that are not as well presented on the infected cell surface during natural infection. By using DNA vaccines that can promote the production of early antigen gene products in mass equivalent to those of the structural genes, T-cells can potentially be "taught" to recognize and destroy infected cells at an early stage by focusing the immune response on viral proteins that are expressed early in infection, before the Class I or Class II presentation is down-regulated by the ongoing natural infection.

Likewise, the antigens and epitopes recognized in chronic herpesvirus infection, during which the virus is continually replicating and shedding structural genes, may not be equivalent to those that would be recognized during acute infection or during initial reactivation. That is, the focus on the T cell epitopes that are dominant during ongoing chronic infection and therefore easily identified, as has also occurred in the HIV vaccine arena, may not be predictive of the epitopes that are important in actual virus control (Letvin *et al.*, 2006). Identification and measurement of the specific immune effectors that limit viral replication after acute infection or reactivation may be much more useful for predicting prophylactic vaccine outcome.

Animal and human experience with DNA vaccines

Many of the studies in larger animal models with DNA vaccines have focused on using this technology to induce cytotoxic T-cells (CTL). Several studies have demonstrated induction of strong CTL responses by parenteral immunization of primates with DNA encoding SIV and/or HIV proteins. In addition, DNA vaccines have been used successfully as priming vaccines in non-human primate models of prime-boost vaccination. Notably, Robinson *et al.* demonstrated that priming with a DNA vaccine containing genes encoding antigen from non-infectious SHIV 89.6, followed by a boost with recombinant modified vaccinia Ankara (MVA) encoding SHIV 89.6 genes, protected 24 of 24 macaques from disease progression following intrarectal challenge with the homologous virus (Amara *et al.*, 2002). Merck and the NIH have used DNA alone or formulated with poloxamers or alum, and then boosted with adenovirus, to protect macaques from SIV challenge (Shiver *et al.*, 2002).

In humans, the immune responses to date have been less robust. In a clinical trial conducted by Hoffman *et al.* a malaria DNA vaccine induced $CD4^+$ or $CD8^+$ T-cell responses in all subjects; however; the responses were not of large magnitude (Wang *et al.*, 2001). In the Merck trials of over 300 subjects to date, approximately 37% of subjects have had measurable gamma interferon positive ELISPOT responses after three or four vaccinations with 5 mg of formulated (aluminum or the poloxamer CRL 1005) or unformulated DNA encoding HIV Gag. (Emini, 2003 Keystone meeting). Although the frequency of response was not 100%, the number of spots induced were similar to the number of T cell responses (both $CD4^+$ and $CD8^+$ T cells) reported by Merck after use of a live attenuated varicella vaccine in the elderly (about 100 spots/million T cells) (Caufield, ICAAC, 2003).

The low response rate in humans to the HIV gag DNA vaccine have been due to the inherently poor immunogenicity of the Gag antigen. This can be hypothesized due to the fact

that canarypox vectors encoding Gag have induced CTL responses in about one third of subjects (Belshe *et al.*, 2001) whereas the same vector encoding CMV pp65 induced CTL responses in 100% of naïve subjects (Berencsi *et al.*, 2001). Similarly, DNA vaccines encoding highly immunogenic antigens such as CMV pp65 may be more effective in inducing robust T cell responses in humans than those reported for the HIV gag DNA vaccine. However, initial data from a bivalent pp65, gB DNA vaccine formulated in the poloxamer CRLI005 also appeared to have an approximate 41% response rate (unpublished viral data.)

While it is well known that DNA vaccination stimulates $CD4^+$ and $CD8^+$ T-cell immune responses in the vaccinated host, the ability to generate a protective humoral response is not as well characterized, especially in humans. Reasonable, but not robust, vaccine-induced antibody levels have been measured in clinical trials of hepatitis B vaccine administered by gene gun (Roy *et al.*, 2000; Letvin *et al.*, 2006). In a recent malaria vaccine trial, DNA immunization primed for increase antibody responses following experimental challenge (S. Hoffman, personal communication). A single dose of DNA, given by electroporation or in an antibody-inducing formulation, is able to protect cats and dogs from rabies challenge for up to one year (Osorio *et al.*, 1999). A DNA vaccine given intramuscularly has generated protective levels of antibody and protected horses from encephalitic West Nile Virus challenge (Davis et *al.*, 2001). In addition, DNA has protected non-human primates from challenge with malaria, influenza, and herpes viruses (Ulmer, 2001). Two vaccines based on induction of antibody are now licensed in animals – a hemorrhagic necrosis virus vaccine for Salmon and a WUV vaccine for horses (Ulmer *et al.*, 2006).

Improving DNA vaccine potency

There are several possible approaches to increasing the potency of DNA vaccines. First, modification of the plasmid DNA vector to increase expression levels has resulted in increased immunogenicity in vivo (Fig. 73.2) (Hartikka *et al.*, 2001; Norman *et al.*, 1997). Changing the nucleotide sequence of certain genes to better reflect preferential codon usage in mammalian cells can result in markedly higher levels of expression in eukaryotic cells *in vitro* and, when incorporated into a DNA vaccine vector, can increase immunogenicity substantially (Andre *et al.*, 1998; Uchijima *et al.*, 1998).

Another general approach to improving DNA vaccines involves adjuvants. Adjuvants can include proteins, small molecule compounds or DNA plasmids encoding immunomodulating proteins, such as cytokines, chemokines and costimulatory molecules. The specific examples are too numerous to list here, but are reviewed elsewhere (Sasaki *et al.*, 1998; Ulmer *et al.*, 2006). We and others have shown that simple mixtures of DNA vaccines with adjuvants are sometimes effective, but appropriate formulation may be required. For example, certain aluminum gels, such as aluminum phosphate, when mixed with DNA vaccines, enhance antibody responses, (Ulmer *et al.*, 1999) while others, such as aluminum hydroxide, inhibit responses as a consequence of electrostatic interaction between the negatively charged DNA and positively charged adjuvant. This detrimental effect can be overcome with appropriate formulation to prevent such binding. We have assessed the effects of cationic lipids, and shown them to enhance the B-cell response without down-regulating the T-cell induction (Hartikka *et al.*, 2001). On the other hand, in our laboratories and others the use of poloxamers has led to an increase in T-cell responses with no detrimental effect on antibodies (Fig. 73.3).

DNA vaccines for specific human herpesviruses

Cytomegalovirus

Genes of interest

The immune response to CMV during acute or chronic infection has been determined by the study of acute and chronic infection in animal models and in man. Antibody appears critical in the prevention of maternal-fetal transmission, and is primarily directed to the envelope glycoproteins, especially gB (Plotkin, 1999). The control of CMV infection in transplant recipients and HIV-infected persons is associated with preserved cellular immune responses, including $CD4^+$, $CD8^+$, and NK T-cells. The $CD8^+$ T cell responses are directed primarily at the immediate early (IE) genes of CMV and at the abundant tegument protein pp65. Approximately 92% of persons have $CD8^+$ responses to pp65 and another 76% to exon 4 of IE1 (Gyulai *et al.*, 2000; Wills *et al.*, 1996). In addition, another one-third of infected individuals have CTL responses to gB. Although pp150 responses have been noted, the degree of these responses are not of the magnitude of those induced by pp65 or IE1.

Almost all infected persons have $CD4^+$ responses to CMV, although the antigen and epitope mapping is not as fully investigated as those for $CD8^+$ T-cells (Kern *et al.*, 2002).

The use of DNA to protect in the maternal fetal situation would likely rely on greater antibody induction, although whether antibodies to gB are sufficient is still debated. An advantage of a DNA approach would be to use polycistronic constructs, such as those encoding the gH/gL/gO or

gM/gN complexes or individual envelope proteins, to elicit antibodies that neutralize via fusion inhibition or other mechanisms.

Animal studies

The plasmid DNA approach to CMV disease has already been validated in a series of recent animal studies. Almost all of these studies have concentrated on the use of gB, the pp65 homologues, or IE1, and in each case have needed to use the species specific strains for the source of the encoded gene (Hwang *et al.*, 1999; Morello *et al.*, 2000; Schleiss *et al.*, 2000; Endresz *et al.*, 1999, 2001). pDNA vaccines encoding murine CMV IE1 and pp65 homologues have elicited immunity associated with 100–1000-fold reduction in viral titers in a mouse model of CMV infection (Ye *et al.*, 2002). In the guinea pig model of maternal-fetal transmission of CMV, DNA encoding gB, but not the pp65 homologue, was capable of partial protection of the newborn pup (Schleiss *et al.*, 2000). (Schleiss, 2004). In addition, the use of pDNA encoding a secreted, transmembrane deleted, form of the gB protein has been shown to elicit higher antibody titers than full length gB in the murine model. (Endresz *et al.*, 2001).

Human trials

Vical Inc. of San Diego moved a poloxamer-formulated bivalent CMV DNA vaccine encoding gB and pp65 into human clinical trials in 2003. The gB gene can be truncated to remove the transmembrane and cytoplasmic domain, thus allowing for greater secretion. The pp65 construct may need modification to remove a published putative kinase encoding region (Gallez-Hawkins *et al.*, 2002). The IE1 constructs being considered for a trivalent vaccine can be engineered to remove both the potential transactivation regions and other biologic activities. A trivalent product that includes the IE1 gene is now in late preclinical development.

The bivalent gB/pp65 vaccine has been tested in both rabbits and mice, and was found to be highly immunogenic for the production of both antibodies (gB) and T cells (pp65). The majority of the vaccine-induced T cell responses in BALB/c mice, as measured in a gamma interferon ELISPOT assay using overlapping peptides encoding the entirety of pp65, are found in the amino terminal half of the protein. The frequency of spot forming cells in mice is higher than that seen with influenza NP, IE1, or similar antigens, when administered on a similar schedule at a similar dose. Studies with the murine pp65 homologue, hCMV pp65, and NP have all shown an improved T cell response using the poloxamer CRL1005. In studies in seropositive, non-vaccinated humans, the gamma interferon positive T cell responses to chronic infection, which may not mirror responses to vaccination, are found to be more frequent in the C-terminal region of the protein, as in contrast to the vaccine induced responses in mice.

Phase 1 studies of this vaccine revealed that three doses of up to 5 mg total DNA induced T cell responses in approximately half of vaccinated individuals. This vaccine has now moved into a Phase 2 study, in which the clinical endpoint is the prevention in CMV seropositive stem cell transplant recipients of detectable CMV viremias after vaccinations of both the donor and the recipient.

Herpes simplex virus 1 and 2

Genes of interest

There appear to be two different approaches to using DNA vaccines in HSV, which may be independently moved forward, or possibly combined. The first would be to increase the antibody response to gB, and/ or gD, or to the gH/gL complex by the use of DNA-augmented by adjuvants, formulations, or cytokines. The second would be to limit viral growth by the recognition and destruction of cells that are in the earliest phases of replication, and prior to the numerous HSV-directed immune evasion mechanisms. In such a scenario, the DNA vaccine would be focused on the early antigens, and those most carefully scrutinized include ICP4, ICP0, and ICP27. ICP 47, which blocks antigen presentation to $CD8^+$ T cells, would be another early antigen for vaccine inclusion, but some biologic modification may be necessary. Other proteins of interest include those that are more abundantly expressed in later stages of virion formation, including the structural proteins VP5, VP13/14, VP16 and VP22.

Animal studies

The concept of direct DNA vaccination for HSV was investigated by a number of different investigators shortly after the discovery of the DNA vaccines (Wolff *et al.*, 1990). In 1994, Rouse *et al.* showed that an *in vitro* model of DNA transfection could be used to generate gB or ICP27-specific CTL from splenocytes (Rouse *et al.*, 1994). Initial reports of low level antibody production and partial protection in mice using gD constructs, (Ghiasi *et al.*, 1995), were followed by more impressive mouse or guinea pig studies using both gB and gD (Bourne *et al.*, 1996a,b; Kriesel *et al.*, 1996; McClements *et al.*, 1996, 1997). The versatility of the DNA approach has been used to study most of the

putative targets of neutralization, including gB, gC, gD, gH/gL or combinations of these (Nass *et al.*, 1998; Cha *et al.*, 2002; Lee *et al.*, 2002). Protection in some experiments using the recurrent zosterform model of HSV appeared to be mediated by T-cells to either gB or ICP27 (Manickan *et al.*, 1995a,b).

Whether these models could be used to predict the need for mucosal versus systemic immunization is not known; however, some studies take advantage of the availability of the DNA constructs in the relapsing guinea pig or acute murine HSV models to test such concepts (Shroff *et al.*, 1999; Eo *et al.*, 2001). In addition, as the results from protection were almost never 100% in early studies, HSV DNA vaccine models have been used extensively to study improvements to the technology, include the use of plasmid or gene modification, (Higgins *et al.*, 2000) alphavirus replicons, (Hariharan *et al.*, 1998) adjuvants, (Bernstein *et al.*, 1999) and cytokine, chemokine or co-stimulatory enhancements (Sin *et al.*, 1998, 1999b; Flo *et al.*, 2000; Sin *et al.*, 2000a,b; Harle *et al.*, 2001; Sin *et al.*, 2001). As in other vaccine systems, the highest titers are often achieved by using a prime-boost approach of initial DNA vaccination, followed by another modality (protein, another vector, or killed virus) (McShane, 2002; Sin *et al.*, 1999a). A DNA prime followed by an inactivated virion was capable of inducing a 4 log reduction in virus shedding in calves that were challenged with bovine herpesvirus-1 (Toussant *et al.*, 2005).

Human trials

No trials of candidate HSV DNA vaccines have been reported in humans despite extensive preclinical work. The relative success of the GlaxoSmithKline vaccine, which used an MPL-based adjuvant to promote a greater T-cell response with Th1 bias, may lead to renewed interest in this approach. Encouraging trial results using replication-defective viruses, such as the DICS-virus, would also be likely to speed the movement of plasmid-based products into humans. Trials of DNA vaccines encoding the early antigens of HSV for use in the therapeutic setting have been planned using gene gun technology, but whether those trials will move forward is not known.

Varicella-zoster virus

Genes of interest

The transmembrane glycoprotein gE, and to a lesser extent gB, contain the major neutralizing antibody targets after VZV natural infection or OKA vaccination. Mapping of CTL targets indicates that immediate early antigens, along with gE, are common (Arvin *et al.*, 2002). The most extensively studied, and first likely target for DNA vaccination is IE62 (Abendroth *et al.*, 1999). There are several biological activities associated with IE62 that may need to be deleted from a vaccine candidate in an attempt to decrease potential transactivation. Other genes that have been suggested to be used as CTL targets in VZV vaccines include IE63 and the thymidine kinase. IE63 is expressed during latency in seropositive subjects, and thus inclusion of this gene in a DNA vaccine may raise concerns about immunopathology (Debrus *et al.*, 1995).

Animal studies

As has been found in CMV and HSV, attempts to augment the generation of a B-cell response have used secreted forms of surface glycoproteins; for example, by removing the transmembrane portions of gE. Publications using a 1.8 kb secreted gE plasmid construct made in the vector pRC/CMV have demonstrated that secreted-gE is expressed very poorly in vitro and elicits suboptimal immunogenicity in mice or guinea pigs (Massaer *et al.*, 1999; Hasan *et al.*, 2002). Normally the glycoprotein gE forms a heterodimer with gI, with gI providing chaperone activity that modulates the trafficking of the gE-gI heterodimer through the golgi and ultimately to display on the cell surface. The lack of gI chaperoning activity may explain the low titers seen in these DNA vaccination studies when injecting the gE plasmid alone. Other studies have shown improvement in titers of gE when administered intradermally by gene gun, although this approach again favored a Th2 bias (Stasikova *et al.*, 2003).

Human trials

No VZV DNA vaccine has moved into the clinic, although the clearest indication would be for use in the immunocompromised setting, in which the use of the licensed live-attenuated vaccine is either contraindicated or not recommended. A bivalent or trivalent vaccine using gE and early antigens formulated in a way to bias toward T-cell over antibody responses is presently a favored approach.

Epstein–Barr virus

Genes of interest

Studies of $CD8^+$ T-cell immune response to EBV have shown that there is often a massive proliferation of T-cells that are narrowly focused on either one or a few MHC

Class I-restricted epitopes (Rickinson *et al.*, 2001). The T-cell response is needed to prevent unchecked viral-driven B cell proliferation. In T-cell deficiency, the emergence of certain EBV-related tumors occurs (for review see else where in this volume), and little role has been observed for antibody-mediated control.

Nonetheless, an antibody-based approach has been used to attempt to prevent or control primary infection, with the majority of investigations focused on the major surface glycoprotein gp350, using Type 1 strains that are most prevalent in Western populations. Little work has been accomplished with other potentially important surface glycoproteins.

For T-cell response induction, initial studies focused on the key early and latency antigens, EBNA1, EBNA2, and LMP1. During the acute infection, T-cells are also rapidly expanded to the lytic antigens, particularly BZLF1, BRLF1 and BMLF1. $CD8^+$ T cell epitopes to most of these genes have been mapped to the HLA-allele for which they are restricted (Rickinson *et al.*, 2001). In persistent infection there is a change in the hierarchy of the genes recognized, with greater responses focused on EBNA3A, EBNA3B, EBNA3C, and LMP2. Since the responses to EBV appear to be narrowly focused, and yet to cover a broad array of HLA haplotypes, this virus may represent an ideal situation to use DNA vaccines based on the polytope approach (Thomson *et al.*, 1998). In such a model, which has been moved to human clinical trials in the HIV vaccine field, multiple epitopes, or epitope dense regions are linked in a plasmid construct using designs that optimize proteosomal cleavage and class I presentation (Livingston *et al.*, 2001). The use of a helper epitope is often employed to improve $CD4^+$ responses and enhance $CD8^+$ T-cell memory.

As with other herpesviruses, it will be necessary to study and potentially modify biologically active elements that may appear to pose safety risk, especially for a known carcinogenic virus. Removal of immune evasion elements could be accomplished for EBNA1 removal of the glycine-arginine repeat (Gar) site responsible for preventing the protein from undergoing degradation by the protesosome (and thus lack of presentation to the Class I system) (Koelle and Corey, 2003).

Animal studies

Studies using DNA vaccines have been quite limited in EBV. A vaccine based on a few HLA-restricted CTL epitopes has been evaluated in mice, but no human studies have been published (Thomson *et al.*, 1998). Studies in mice have shown the expected immunogenicity using DNA constructs encoding gp350 (Jung *et al.*, 2001).

Human trials

No trials of DNA vaccines have been undertaken in EBV.

Summary

DNA vaccination, as with any new emerging technology, has passed through a series of unreasonable positive expectations, and has suffered at the hands of poorly defined negative criticisms. Although no definitive human efficacy data exist, however, the technology is only a little more than a decade old. The results in human trials to date have been encouraging, but suboptimal, and may have suffered form use of first generation vectors, lack of formulations, and poorly selected immunogens and disease states. Human herpesvirus diseases represent a frontier in which this technology is likely to prove whether it can live up to its enormous promise and potential. If initial trials in CMV or HSV show promise in the coming two years, renewed enthusiasm for moving this approach into humans with the remaining herpesviruses should follow.

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Adoptive immunotherapy for herpesviruses

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Introduction

Herpesvirus infections rarely cause significant problems in the immunocompetent human host. However, in the immunosuppressed, for example, recipients of hematopoietic stem cell transplants (HSCT) (Rooney *et al.*, 1998), solid organ transplants (SOT), or human immunodeficiency virus (HIV)-infected individuals, viral infections/reactivations are common and are associated with considerable morbidity and mortality. The resultant uncontrolled infections correlate with a lack of cellular immunity against viral antigens (Weinberg *et al.*, 2001). While effective antiviral drugs are available for the treatment of some herpesvirus infections, adoptive immunotherapy, which is the artificial reconstitution of virus-specific T-cells with *in vitro* expanded cytotoxic T-lymphocytes (CTLs), for the prophylaxis and/or treatment of herpesviruses is an attractive option. The γ-herpesvirus, Epstein–Barr virus (EBV) is also associated with a heterogeneous range of malignancies and diseases that occur in apparently immunocompetent individuals and since these malignancies also express "foreign" viral antigenic targets they may also be good candidates for immunotherapy (Rickinson and Kieff, 2001). The advances in such adoptive immunotherapeutic approaches will be discussed in this chapter.

Therapy for herpesvirus-related infections and diseases

Infectious complications relating to herpes simplex virus (HSV), varicella zoster virus (VZV) (Asanuma *et al.*, 2000), Kaposi's sarcoma virus (KSV) (Wang *et al.*, 2000), human herpesvirus (HHV)-6, -7 (Clark, 2002; Clark *et al.*, 2003; Clark and Griffiths, 2003), cytomegalovirus (CMV) (Michaelides *et al.*, 2002) and EBV (Heslop *et al.*, 1994) are common in immunocompromised individuals. In particular, during allogeneic HSCT and SOT, conditioning regimens involving a combination of immunosuppressive drugs and radiation therapy eliminate the recipient immune system. T-cell depletion, delayed T-cell recovery *in vivo*, and prolonged immunosuppressive treatments are all associated with a significant risk of herpesvirus reactivations post-transplant. In the HSCT setting, immune recovery occurs after engraftment and expansion of the recipient immune system, in vivo (Weinberg *et al.*, 2001). However, following SOT the patient remains in an immunosuppressed state lifelong, and thus the recipient immune system can never achieve its full potential. Treatments for herpesvirus infections include vaccination approaches, which aim to evoke an immune response by administration of an immunogen, as a peptide or DNA, directly into patients. However, in the immunocompromised host, vaccines may not function optimally. Anti-viral drugs, such as acyclovir and ganciclovir inhibit productive virus replication in vivo, and can effectively control α- and β-herpesvirus infections, in which disease is associated with the lytic life cycle. However in the case of CMV, antiviral drugs appear to alter subsequent reactivations such that antiviral T-cells recover less effectively, leading to a late, chronic CMV-reactivation, particularly in association with chronic graft vs. host disease (GvHD) (Junghanss *et al.*, 2002; Nguyen *et al.*, 1999). In addition, drug resistant escape mutants have been reported (Springer *et al.*, 2005; Razonable and Paya, 2003).

Most antiherpes viral agents are prodrugs that are activated by lytic cycle kinases, and thus have limited efficacy against γ-herpesviruses, such as EBV, whose pathology is associated with its latent cycle. EBV-associated lymphoproliferative disease (LPD) in which virus-transformed

Table 74.1. Human herpesviruses

Herpesvirus subgroup	Virus	Tumor association
alpha	HSV-1	none
	HSV-2	none
	VZV	none
beta	CMV	none
	HHV-6	none
	HHV-7	none,
gamma	EBV	Burkitt's lymphoma, Gastric carcinoma, Nasopharyngeal carcinoma, Hodgkin's disease, Post-transplant lymphoproliferative disease, T-cell lymphoma, Leiomyosarcoma
	HHV-8	Kaposi sarcoma of HIV positive and negative patients, body cavity lymphoma, Castleman's disease

B-cells proliferate in an uncontrolled manner, has emerged as a significant complication of both HSCT (d'Amore *et al.*, 1991) and SOT (Straathof *et al.*, 2002; Savoldo *et al.*, 2001). EBV is also linked with a number of tumors that are not associated with iatrogenic or intrinsic immunosuppression including Hodgkin's disease (HD), nasopharyngeal carcinoma (NPC), and Burkitt's lymphoma (BL). (Rickinson and Kieff, 2001.) A recent advance in the treatment of EBV-LPD in HSCT recipients has been the humanized anti-B cell monoclonal antibody, rituximab, designed for the treatment of follicular lymphoma, but which has proved effective for the treatment of EBV positive B-cell lymphomas (Kuehnle *et al.*, 2000). However, rituximab has little efficacy against HD, which rarely express CD20, or NPC (Table 74.1).

The potential for using in vitro expanded virus-specific CTLs as either prophylaxis or treatment for viral infections/reactivations stemmed from the observation that viral reactivations post-transplant were clearly associated with the lack of recovery of virus-specific T-cells. Donor leukocyte infusions (DLIs) had therapeutic benefits for the treatment of EBV positive lymphomas occurring after HSCT (Papadopoulos *et al.*, 1994). However, GvHD resulting from the presence of alloreative T cells was a common side effect, and a pure population of virus-specific cells was predicted to be safer and therapeutically beneficial. Since CMV- and EBV-associated diseases remain potentially fatal complications in the immunosuppressed these viruses have been the focus of adoptive immunotherapy approaches. A variety of strategies have been developed, some of which have been translated into the clinic and assessed for safety and efficacy and a number of these will now be reviewed.

Cytomegalovirus

As many as 80% of healthy individuals show evidence of past CMV exposure and persisting humoral and cellular immunity. The virus remains latent in cells of the myeloid lineage but reactivation from their resting state occurs sporadically, (Mocarski and Courcelle, 2001; Pass, 2001). Analysis of the T cell immune response to individual CMV proteins has indicated that there is a hierarchy of immunodominance, i.e. certain proteins tend to be strong targets of T-cell immunity while other proteins are subdominant. In the case of CMV the bulk of the CD8+ T cell immune response is directed against two immunodominant proteins, pp65 and IE (Bunde *et al.*, 2005; Kern *et al.*, 2000). pp65 is a matrix protein produced during the late phase, and it is thought that during primary infection, the initial viral input dominates the immune response, since the presentation of subsequently expressed viral proteins to the immune response is inhibited. Conversely, reactivation of virus from a resting state may result in the formation of T-cell memory dominated by the immediate early (IE) protein, which is transcribed prior to the initiation of viral immune evasion strategies. Thus IE may also be an important target for CTL infusions.

Epstein–Barr virus

The γ-herpesvirus, EBV, establishes latent infections in target B-lymphocytes, (Rickinson and Kieff, 2001; Rickinson and Moss, 1997)). In type III latency the eight virally encoded latent cycle proteins; nuclear antigens EBNAs 1, 2, 3A, 3B, 3C, -LP and latent membrane proteins LMPs 1 and 2, along with two small non-polyadenylated RNAs (or EBERs) and the BamHI A RNAs cause continuous B-cell proliferation resulting in EBV-transformed lymphoblastoid cell lines (LCLs). Latency types I and II demonstrate a more restricted pattern of latent gene expression. Latency II was first described in EBV-positive Hodgkin's disease where the EBERs, the BamHI A RNAs, EBNA1, and LMP1 and 2 are expressed. EBV-associated nasopharyngeal carcinoma (NPC) also expresses a latency II pattern of gene expression. Latency I was first described in EBV-positive Burkitt's lymphoma (BL) lines, which express EBERs, the BamHI A RNAs, and EBNA1 (Rickinson and Kieff, 2001). Note that EBNA1 is the only viral protein expressed in all the different forms of malignancy-associated latency, and in each type of latency a different transcriptional promoter is used for EBNA1 expression (Amyes *et al.*, 2003). This protein is absolutely required for episomal maintenance of the viral genome. However, in a cell, which is not cycling, it is thought

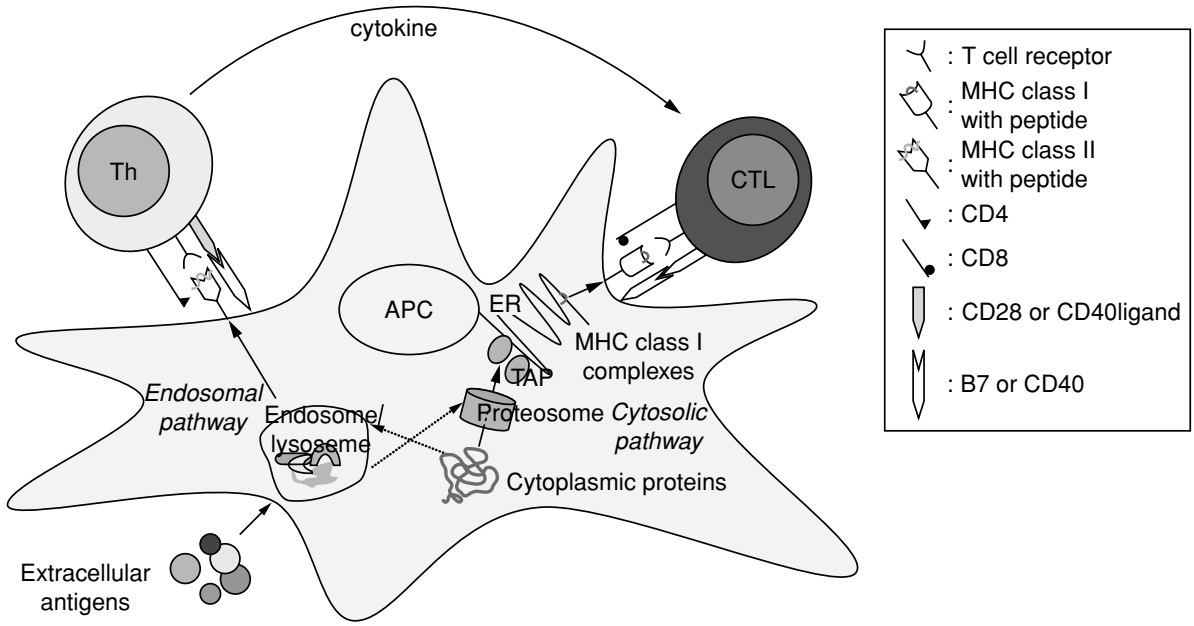

Fig. 74.1. Generation of cell-mediated immune response. (See color plate section.)

that virus persists without the expression of EBNA1, and only LMP2 can be detected in EBV-infected resting B-cells (type 0 latency) (Qu and Rowe, 1992; Tierney *et al.*, 1994; Miyashita *et al.*, 1997).

Initial work identifying the targets of the strong specific memory T-cell response in healthy seropositive donors demonstrated the dominance of reactivities against early lytic cycle proteins and the latent antigens EBNA3A, -B, and –C in the majority of individuals tested, restricted through a wide range of MHC class I alleles. LMP2 is also a common target for specific T-cells but reactivity is generally weak and of low frequency, when compared to that of the immunodominant antigens. In contrast, only rare reactivities specific for epitopes derived from EBNA1, EBNA2, EBNA-LP, and LMP1 have been identified (Rickinson and Moss, 1997). Memory T-cells specific for the latency-associated antigens persist throughout the lifetime of the normal healthy host and the frequency of CTL precursors directed against different viral epitopes remains relatively stable within an individual (Hislop *et al.*, 2002; Amyes *et al.*, 2003).

T-cell activation

The design of successful immunological strategies to treat human virus-associated diseases and malignancies requires an understanding of the effector processes that control viral infection and the mechanisms viruses use to evade such responses.

Virus-specific CD4+ Th cells and CD8+ CTLs mediate the effector mechanisms necessary to resolve acute infection as well as providing immune memory, which protects against re-exposure to acute virus infection and controls the reactivation of latent viruses (Abbas *et al.*, 1996). CD8+ CTLs recognize virus-infected cells through interaction of their T-cell receptor with virus-derived peptides bound to the major histocompatibility complex (MHC) class I molecule of the infected cell. Viral proteins endogenously synthesized in the infected cell are degraded into short antigenic peptides by the cellular antigen-processing machinery, carried to the cell surface by MHC molecules, where they are presented to T-cells circulating through lymphoid tissues. These peptides are generally 8–10 amino acids long, generated within cells by a cytoplasmic proteolytic complex known as the proteosome and then transported into the endoplasmic reticulum by TAP (transporters associated with antigen processing), where they are complexed with MHC class I molecules for cell surface presentation (Jondal *et al.*, 1996; Pamer and Cresswell, 1998). Since virtually all nucleated cells express MHC class I, any virus-derived protein may be presented by MHC class I molecules and should be susceptible to subsequent recognition by specific CD8+ T-cells. A role for CD8-mediated viral control has clearly been documented in animal models, further supported by the identification of a number of mechanisms whereby viruses have evolved ways of evading the MHC class I antigen presentation pathway (McMichael, 1998) (Fig. 74.1).

For activating (priming) naïve CTL precursors, antigenic peptides must be presented by professional APCs, which also provide the necessary co-stimulatory signals (i.e., interaction of B7 or CD40 on APC with CD28 or CD40L on T-cells, respectively) (Sigal *et al.*, 1999). If the T-cell receptor is engaged without costimulatory signals, T-lymphocytes can become anergized. However, activated CTLs do not need costimulatory molecules to exert their effector functions, namely, cytolysis or induction of apoptosis of the target cell. Importantly, target cells frequently do not express co-stimulatory molecules. CMV and EBV are interesting exceptions in which latency occurs in professional APCs.

CD4+ T-cells play a role in antibody responses and also recognize antigens that are phagocytosed from an exogenous source, and processed and presented in the context of MHC class II molecules. Only APCs that are MHC class II-positive can activate CD4+ T-cell precursors. CD4+ T-cells also play a role in anti-viral immunity; activated Th cells produce a variety of cytokines including IL-2, IFN-γ, and TNF-α, which have direct anti-viral activity. Simultaneously, these activated T-cells also serve to condition APCs to activate virus-specific CTLs. When the infected cell is MHC class II positive, CD4+ T-cells can also be cytolytic to the target cells and this function has been clearly demonstrated for herpesvirus infections including CMV, EBV, HSV, and VZV (Lanzavecchia, 1996; Borysiewicz and Sissons, 1994).

Strategies for producing T-cells for adoptive immunotherapy

The generation of virus-specific CTLs or T-cell clones for adoptive immunotherapy is an attractive alternative to anti-viral drug therapy as infused CTLs have the potential to persist in vivo without related toxicity. Prior to using in vitro expanded CTLs for infusion purposes the cells must fulfil a number of requirements.

(i) All cell preparation must be carried out under good manufacturing practices (GMP).
(ii) CTL lines or T cell clones must be virus-specific and lack reactivity with recipient alloantigens or self-antigens.
(iii) CTL lines/clones should include both specific CD4+ and CD8+ T-cells.
(iv) Cells for infusion must be present in sufficient quantities for safety testing and infusion.

To meet these requirements the choice of antigen and APC to be used for activation and expansion purposes must be carefully chosen, and immunological and virological monitoring of patients post-infusion is desirable to demonstrate the safety and efficacy of T-cell therapy. Since immunodominant T-cell target antigens and sources of APCs have already been identified for both CMV and EBV, and related infections constitute a significant problem in the immunocompromised human host, initial adoptive immunotherapeutic strategies focused on these two herpesviruses.

Adoptive immunotherapy for cytomegalovirus

A number of factors are predictive of CMV infection post-HSCT transplant including:

(i) receiving a graft from an HLA-mismatched or unrelated donor,
(ii) recipient CMV seropositivity coupled with donor CMV-negativity,
(iii) the use of submyeloablative or reduced intensity, highly immunosuppressive conditioning regimens.

It has been shown that recovery of CD8+, CMV-specific CTLs in the early post-transplantation period inversely correlates with the development of CMV-related disease, and it has been reported that, in up to 65% of patients, CMV-specific CTLs do not develop during this period, leaving this cohort at high risk of virus-related complications (Pass, 2001). In a pioneering study by Walter and colleagues, CD8+ CMV-specific T cell clones were isolated and expanded from the blood of bone marrow donors and administered to 14 patients prophylactically at weekly intervals in doses escalating from 3.3×10^6/kg to 1×10^9/kg, beginning 30–40 days post-transplant (Walter *et al.*, 1995). The infused clones were reactive against CMV virion proteins, including the immunodominant T-cell target, pp65. Careful monitoring of the status of CMV-specific immunity pre-infusion indicated that 11 of the 14 donors lacked any evidence of anti-viral activity pre-infusion, while after the first infusion responses were detected in all donors. The magnitude of these responses increased with successive injections but specific CD8+ T-cell immunity did not persist in patients who did not have a concurrent recovery of CD4+ T-cells, highlighting the importance of Th cells in the maintenance of anti-viral activity *in vivo*. In a number of infused recipients the authors could directly correlate CMV T-cell immunity and the persistence (for up to 12 weeks) of transferred T-cells by following rearranged Vα and Vβ genes for the T-cell receptor (TCR) as molecular markers. Neither CMV viremia nor disease developed in any of the treated patients (Walter *et al.*, 1995).

A second CMV immunotherapy trial was published by Einsele and colleagues in 2002 (Einsele *et al.*, 2002). Polyclonal CMV-specific CTL lines were infused into eight HSCT recipients who had persisting or recurring CMV infection despite the prolonged use of anti-viral medications. Donors were CMV sero-positive and included HLA-matched

siblings, HLA-matched unrelated donors, or HLA mismatched related or unrelated donors. At the time of the first CTL infusion, seven of the eight patients treated were still receiving some form of immunosuppression as prophylaxis for GvHD.

Polyclonal CTL lines were prepared by stimulating PBMCs with CMV lysate, which is a source of multiple CMV antigens that could be phagocytosed and presented by peripheral blood monocytes. The CTLs were expanded using autologous, irradiated PBMC feeder cells to present CMV lysate, and IL-2. A total T-cell dose of $1 \times 10^7/m^2$ was administered without toxicity. In all cases the lines were predominantly CD4+ and CMV specificity was confirmed prior to infusion using proliferation assays. The advantage of this method is that CTL lines containing both CD4+ and CD8+ T-cells with a broad range of antigen and epitope specificities could be induced and infused. The efficacy of CTL infusions was assessed using immunological assays to measure the functional capabilities of PBMCs drawn from patients at two-weekly intervals postinfusion and viral load was assessed using quantitative or semi-quantitative PCR analysis.

Following T-cell therapy, six of the eight patients, all of whom lacked anti-CMV reactivity before adoptive transfer, responded in vitro to CMV protein and had no detectable levels of CMV in PBMCs. Viral load as determined by quantitative PCR showed significant reductions following therapy in 7/7 evaluable patients. This reduction in viral load was persistent in five, and transient in two patients. Two patients who did get a CMV reactivation received a course of intense immunosuppression for GvHD around the time of T-cell therapy. Immunological monitoring supported the assertion that infusion of virus-specific CD4+ T-cell lines hastened the *in vivo* recovery of virus-specific CD8+ T-cells, as significant expansions of peptide-specific CD8+ T-cells, detectable by tetramer, were discernible within 2 months of therapy.

This trial confirmed the efficacy of T-cell therapy, either as prophylaxis or treatment, for CMV infection and disease post-HSCT. It also provided a simple and rapid means to reconstitute long-term CD4 and CD8 CMV-specific immunity using CTL doses >2 logs lower than that used in the previous study.

Despite the positive outcome of both these trials there were also some associated difficulties. In the case of Walter and colleagues the methodology used for CTL clone generation required a skin biopsy for the generation of dermal fibroblasts for use as APCs. In addition, live virus was used to stimulate specific CD8+ T-cell reactivity. From a therapeutic point of view, each T-cell clone can only be specific for one epitope peptide from one antigen. Therefore the breadth of reactivities generated for infusion may be lacking in some donors depending on the number of different clones infused, and the paucity of CD4+ cells probably affected the persistence of these cells in vivo. Finally, from a practical point of view the generation of large numbers of specific T-cell clones for infusion is time consuming, technically difficult, and expensive.

In the second study, CTL lines could be generated for only 68% of patients, suggesting that CMV CTL generation using PBMCs pulsed with CMV lysate may be a suboptimal production method. Further optimization, perhaps by using professional APCs, may improve the overall percentage of successful lines generated. Both studies reported poor results achieved in patients receiving immunosuppressive therapy to counteract GvHD, illustrating one potential barrier to effective immunotherapy for HSCT recipients in the early post-transplant period.

Adoptive immunotherapy for EBV post-transplant lymphoproliferation disease

The majority of EBV-PTLD in HSCT recipients are of donor B-cell origin. Malignant B-cells usually express the complete panel of latent viral antigens, as well as abundant co-stimulatory molecules. Thus, they are highly immunogenic, and in healthy individuals are eliminated by circulating EBV-specific CTL. The incidence of EBV lymphoproliferation in high-risk patients, i.e. those receiving a T-cell depleted transplant from an unrelated donor or an HLA-mismatched, related donor, ranges from 1% to 25% (Curtis *et al.*, 1999). By contrast, removal of B-cells from the graft decreases the incidence to less than 2%, suggesting that uncontrolled EBV-driven lymphoproliferation may be favored when the ratio of T-: B-cells is severely disrupted (Hale and Waldmann, 1998). An increase in EBV DNA in peripheral blood, measured by quantitative real time PCR is used to predict the development of EBV-LPD, with several studies confirming that EBV load correlates with an increased risk of LPD (Rooney *et al.*, 1995a,b; Stevens *et al.*, 2001; van Esser *et al.*, 2002; Wagner *et al.*, 2003).

EBV-associated PTLD is a good target for immunotherapy since most donors are seropositive, and EBV-specific CTLs can readily be reactivated from PBMC using LCLs as APCs for stimulation and expansion. LCLs are easily made by infecting donor PBMCs with a laboratory strain of EBV, resulting in the outgrowth of B-cell lines expressing target antigens identical to those expressed in LPD. Since 1993, our group has infused over 60 stem cell recipients with donor-derived EBV-specific T-cell lines. 2×10^7CTL/m^2, was established as a safe and efficacious dose for both

prophylaxis and treatment (Rooney *et al.*, 1995a,b 1998a,b). In each case the CTL lines were polyclonal, with CD4:CD8 ratios ranging from 2:98 to 98:2. The first 26 patients enrolled in this study received CTLs which were genetically marked with a retroviral vector containing the neomycin resistance gene (*neo*), allowing the collection of data about the persistence and localization of infused cells in vivo. In addition, 6 patients received virus-specific CTL after the onset of lymphoma.

None of the patients treated with EBV-specific CTL as prophylaxis developed PTLD, in contrast with an incidence of 11.5% in a historical untreated control group (Heslop and Rooney, 1997). At study entry 9 patients had elevated EBV–DNA levels, which is highly predictive of the development of LPD. Analysis of EBV-DNA levels postinfusion showed direct evidence of anti-viral activity as DNA levels decreased by up to four logs within 1–3 weeks of the first T-cell infusion. In patients who received marked cells, specific CTLs could be detected for up to 78 months post CTL (Rooney *et al.*, 1998a,b).

Although CTL had proven effective at decreasing viral load, it was uncertain whether infusions of T-cells could be successful in treating an already established lymphoma. We treated a total of six patients with evident lymphoma and in five of the six patients complete remission was achieved. In the remaining patient, comprehensive in vitro characterization revealed the increased dominance of a virus deletion mutant after CTL infusion (Gottschalk *et al.*, 2001). The donor CTL line generated by co-culture of donor PBMCs with an LCL generated using the B95-8 virus strain revealed epitope reactivity directed predominantly against the immunodominant EBNA3B antigen, and specifically against two immunodominant epitope peptides within this antigen; namely AVFDRKSDAK (AVF), aa 399–408 and IVTDFSVIK (IVT), aa 416–424, both recognized in the context of the HLA A*11 allele (Gottschalk *et al.*, 2001). Sequence analysis of the resident patient tumor revealed two resident viruses. One harbored a deletion in the EBNA3B gene, which removed the AVF and IVT peptide sequences. Since the virus with the wild type EBNA3B could no longer be detected after CTL infusion, the CTL line had reduced ability to recognize the mutant virus. This is a concern for all adoptive immunotherapy protocols as this case illustrates that, even in a polyclonal system, a mutation in a tumor-specific antigen can ultimately result in tumor escape.

In total over 160 patients received CTLs, most as prophylaxis and 6 as treatment for established lymphoma. In one patient who received CTL for treatment of bulky nasopharyngeal disease, the infusion caused an inflammatory response which required mechanical ventilation. However the child made a full recovery and achieved complete remission (Straathof *et al.*, 2005). The authors were unsuccessful in generating a CTL line for two eligible patients whose donors were EBV-seronegative. An additional problem with CTL use is that the total time for LCL and CTL production and testing is approximately 12 weeks, while the aggressiveness of this lymphoma means that CTL should be administered prophylactically for high-risk patients. Despite this, in the absence of effective antiviral agents, adoptive immunotherapy was an attractive alternative as EBV-specific CTLs are safe and effective at controlling and treating EBV in high-risk patients post-transplant. More recently, monoclonal anti-B-cell antibodies such as rituximab demonstrated overall response rates of 69%–100% when used as a treatment for LPD post HSCT (Kuehnle *et al.*, 2000). Since rituximab is now widely available, it has now largely replaced CTL as the preferred treatment of EBV after HSCT. In the 6 months usually required for B cell recovery after treatment, endogenous antiviral immunity recovers and is able to control EBV when it reappears.

Adoptive immunotherapy for EBV post-solid organ transplant

Although it has been shown that adoptive immunotherapy is an effective treatment of EBV-LPD in the HSCT recipient, the optimal treatment for patients receiving solid organ transplants has yet to be established. In this cohort, LPD is usually of recipient origin, thus the preparation of EBV-specific CTLs requires the use of autologous or HLA-matched cells. Autologous EBV-specific CTL can be generated from the peripheral blood of SOT patients prior to transplant (Haque *et al.*, 1998), but a number of groups have also reported the successful generation of virus-specific CTL lines from patients receiving immunosuppression, including those with overt lymphoma, showing that EBV-specific CTL persist, but are unable to function in vivo (Straathof *et al.*, 2002; Savoldo *et al.*, 2001; Khanna *et al.*, 1999; Comoli *et al.*, 2002). Ten SOT patients with high viral load received autologous EBV-specific CTLs and eight showed a subsequent normalization of EBV–DNA levels and an increase in virus-specific CTL precursors (Haque *et al.*, 1998; Comoli *et al.*, 2002). Until recently, the persistence of infused CTL had not been examined (Savoldo *et al.*, 2001; Comoli *et al.*, 2002). Savoldo and colleagues have investigated the in-vivo safety, efficacy and persistence of autologous EBV CTL for the treatment of SOT recipients at high-risk for EBV-associated PTLD (Savoldo *et al.*, 2006). Twelve SOT recipients at high risk for PTLD,

or with active disease, received autologous CTL infusion without toxicity. Real-time PCR monitoring of EBV-DNA showed a transient increase in plasma EBV-DNA suggestive of lysis of EBV-infected cells, although there was no consistent decrease in virus load in peripheral blood mononuclear cells. Interferon-γ Elispot assay and tetramer analysis showed an increase in the frequency of EBV-responsive T cells, which returned to pre-infusion levels after 2–6 months. None of the treated patients developed PTLD. One patient with liver PTLD showed a complete response, and one with ocular disease had a partial response stable for over one year (Savoldo *et al.*, 2006). These data are consistent with an expansion and persistence of adoptively transferred EBV-CTL, that is limited in the presence of continued immunosuppression, but that nonetheless produced clinically useful anti-viral activity.

Adoptive transfer of EBV-specific CTL for Hodgkin's lymphoma

Having shown that EBV infected cells, which express a wide range of EBV encoded antigens, are susceptible to immunotherapy our group is now evaluating if the malignant cells of Hodgkin's disease, which express a more restricted pattern of antigens, are also targets for this approach. In a Phase I dose escalation study we have evaluated the use of autologous EBV-specific CTL for patients with EBV-positive Hodgkin's disease (Roskrow *et al.*, 1998). We treated eight patients with relapsed Hodgkin disease with two infusions ($2 \times 10^7/m^2$–$1.2 \times 10^8/m^2$) of EBV-specific CTL. In seven of these patients the CTL were retrovirally marked. Increases in anti-viral immunity, and decreases in virus load demonstrated the in vivo biological activity of the infused CTL and gene-marked T cells could be detected for up to 9 months. Trafficking to tumor sites was demonstrated in two patients by in situ hybridization to the *neo* marker gene in mediastinal tumor tissue and by PCR in a malignant pleural effusion. Further, partial tumor responses were observed. However, no patient with bulky disease was cured. In a second group, treated with CTL as adjuvant therapy after autologous stem cell rescue, four of five patients remain in remission over 24 months after CTL infusion, including one patient who had residual disease after HSCT and prior to CTL infusion.

Although these results have been promising the antiviral responses were transient, and no patient with aggressive relapsed Hodgkin's disease has been cured. This may be due to a lack of specificity of the EBV-specific CTL for the subdominant LMP1 and LMP2 antigens present on the Hodgkin's tumor. Using dendritic cells transduced with LMP2 to stimulate and expand the CTL, LMP2-specific CTL have been generated that have an increased cytolytic activity to LMP2 positive targets in vitro when compared to EBV-CTL (Gahn *et al.*, 2001; Rooney *et al.*, 2002; Bollard *et al.*, 2004). A clinical trial using LMP2-specific CTL for the treatment of Hodgkin's and non-Hodgkin's lymphoma expressing a type II latency has recently begun.

Adoptive transfer of EBV-specific CTL for nasopharyngeal carcinoma

Nasopharyngeal carcinoma is a malignant disease with a variable range of incidence depending on age, geographical location, race, and EBV exposure. It has an annual incidence of nearly 1 case per 100 000 children <21 yrs in the USA (Niedobitek, 2000). The non-keratinizing NPCs are uniformly associated with EBV. Despite the good overall survival rates, particularly in children, current NPC treatment regimens including radiotherapy and chemotherapy are still far from ideal. Follow-up reports have shown increased risks for treatment-related morbidity and mortality (Pao *et al.*, 1989). Late medical complications after treatment for NPC include growth hormone deficiency, hypothyroidism, pulmonary fibrosis, and secondary malignancies. It is therefore, desirable to develop novel therapies that could improve disease free survival in relapsed/refractory patients and which might ultimately reduce the incidence of long-term treatment related complications in all patients.

Chua *et al.*, treated four patients with advanced nasopharyngeal carcinoma with 5×10^7–3×10^8 autologous EBV CTL (Chua *et al.*, 2001). Although it was difficult to confirm improved tumor control in these patients who had significantly bulky disease, the treatment was safe and elevations in CTL precursor frequency were seen. In 3 patients, host surveillance of EBV replication was restored resulting in a reduction in the plasma EBV burden. Straathof and colleagues have treated 10 patients with advanced NPC with autologous CTLs. All patients tolerated the CTLs, although one developed increased swelling at the site of pre-existing disease. At 19 to 27 months after infusion, 4 patients treated in remission from locally advanced disease remain disease free. Of 6 patients with refractory disease prior to treatment, 2 had complete responses, and remain in remission over 11 to 23 months after treatment; 1 had a partial remission that persisted for 12 months; 1 has had stable disease for more than 14 months; and 2 had no response. These results demonstrate that administration of EBV-specific CTLs to patients with advanced NPC is feasible, appears to be safe,

and can be associated with significant antitumor activity (Straathof *et al.*, 2005).

For EBV+ve tumors, such as HD and NPC, which arise in patients with an intact immune system, approaches that overcome immune evasion strategies used by these immunogenic tumors may be required. Further improvements in the *in vivo* function and persistence of CTL may require genetic modification of CTL lines to provide resistance to tumor-derived immunosuppressive chemokines and cytokines (Bollard *et al.*, 2002; Foster and Rooney, 2006). Alternatively, modifications to the host or tumor environment may be sufficient (Dudley *et al.*, 2002).

Multivirus-specific CTL lines

Since both EBV and CMV reactivations are common side effects post-transplant, some groups have considered generating bi-virus specific CTLs for infusion purposes (Sun *et al.*, 1999, 2000). Lucas and colleagues carried out a phase I/II clinical protocol for the generation and infusion of EBV and CMV-specific CTL, initiated by culturing donor-derived PBMC with irradiated donor-derived LCL transduced with a retrovirus expressing pp65 (Lucas *et al.*, 2000). Two further stimulations with the transduced LCL were performed before assessing the CMV and EBV-specific cytolytic activity of the line. Clinical data is not yet available from this study. Our group has also generated multivirus-specific CTL lines for therapeutic purposes by genetically modifying APCs to enable production of $CD4^+$ and $CD8^+$ T lymphocytes specific for CMV, EBV, and multiple serotypes of adenovirus from a single cell culture. Eleven multivirus-specific CTL lines were administered as prophyaxis or treatment to immunocompromised patients. In all cases the single T lymphocyte line was able to expand in vivo into multiple discrete virus-specific populations that could supply clinically measurable antiviral activity, and all patients with evidence of active CMV, EBV or adenoviral infection had a relatively rapid reduction in viral titer and resolution of disease symptoms, which coincided with the expansion of virus-specific CTL. Therefore, we can conclude that monoculture-derived multispecific CTL could provide a safe and efficient means to restore virus-specific immunity in the immunocompromised host (Leen *et al.*, 2006b).

CMV and EBV immunotherapy in HIV positive individuals

Adoptive immunotherapy approaches have been applied to the treatment of acquired immunodeficiency syndrome (AIDS) where pathogenicity is synonymous with the depletion of CD4+ T-lymphocytes, and the consequent increased susceptibility to infections, either from *de novo* exposure or reactivation of latent viruses such as EBV (Riddell *et al.*, 1996). To date, no group has targeted the herpesvirus infections/reactivations that occur as a result of HIV-induced immunosuppression. Brodie and colleagues have assessed the safety and efficacy of expanding and transferring up to 3.3×10^9 cells/m^2 autologous HIV-1 specific, Gag-specific CD8+ T-cell clones to HIV-infected individuals, and reported the accumulation of the transferred cells at sites adjacent to HIV-infected cells in the lymph nodes (Brodie *et al.*, 1999; Brodie *et al.*, 2000). In addition, levels of circulating productively infected CD4+ T-cells transiently decreased. However within 7 days of infusion the percentage of productively infected cells had returned to pre-infusion levels, suggesting that adoptive immunotherapy may not be efficacious in this patient cohort. Since the advent of highly active antiretroviral therapy (HAART) the incidence of EBV+ve CNS lymphomas has decreased. Strangely and perhaps controversially however, the incidence of other EBV+ve lymphomas has not (Levine *et al.*, 2001). Since the overall health and life expectancy of patients with HIV has improved with HAART, the potential for adoptive transfer of herpesvirus-specific T-cells in these patients should perhaps be revisited.

Alternative approaches for activating virus-specific CTLs

In the outlined adoptive immunotherapy approaches there are a number of differences in terms of the phenotype of the infused cells, the choice of antigen for activation, the APC utilized to stimulate T-cells, the CTL expansion methods, and also the numbers of cells that are infused. The relative advantages and disadvantages of these methods, as well as alternative approaches which may be attempted in future studies, will be discussed.

Clones vs. polyclonal lines

The original Riddell CMV immunotherapy protocol, which proved efficacious in vivo, relied on the infusion of large numbers (3.3×10^6 cells/kg to 1×10^9 cells/kg) of CD8+ T-cell clones (Walter *et al.*, 1995). In contrast, infusion of smaller numbers (1×10^7–2×10^7/m^2) of polyclonal, virus-specific CD4+ and CD8+ CTL lines proved efficacious in the context of CMV and EBV prophylaxis and/or treatment in the transplant and tumor setting, suggesting that a mixed population of CD4+ and CD8+ T-cells rather than

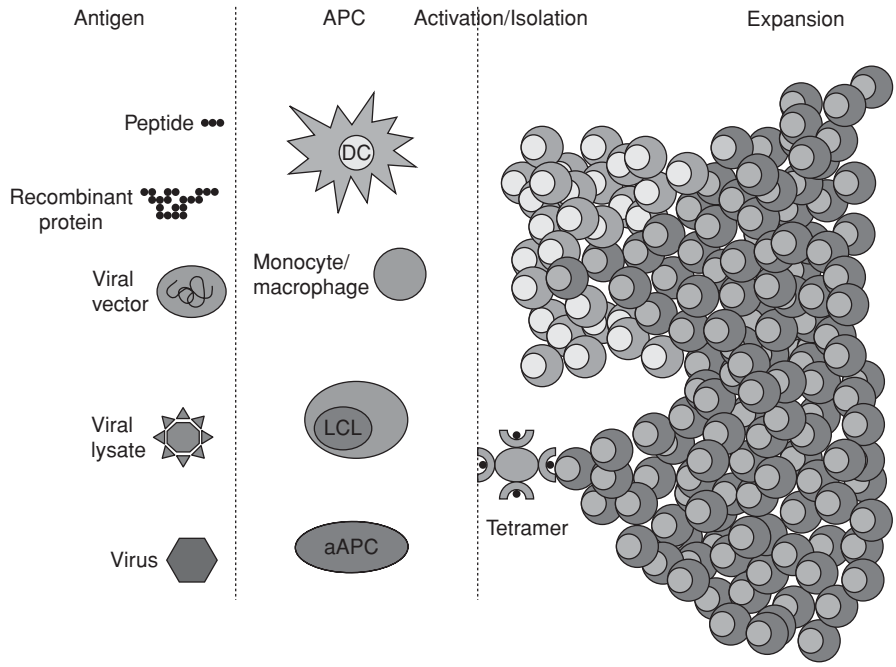

Fig. 74.2. Generation of antigen-specific T-cells.

the quantity of cells is the important factor for infusion purposes in situations where the recipient immune system has been depleted (Einsele *et al.*, 2002; Rooney *et al.*, 1995a,b; Peggs *et al.*, 2003).

Antigen

There are a number of options when choosing a source of antigen to stimulate an immune response. Riddell and colleagues used live CMV, (Walter *et al.*, 1995) and Einsele *et al.* utilized CMV lysate to stimulate CMV-specific immunity, (Einsele *et al.*, 2002) while Rooney and colleagues used an EBV-transformed B-cell line expressing EBV lytic and latent antigens to stimulate an immune response (Rooney *et al.*, 1995a,b). Alternative sources of antigen, which have been investigated in *in vitro* studies, include live or inactivated virus, recombinant protein, replication-defective viral vectors expressing one or more immunodominant T cell target antigens (Bonini *et al.*, 2001), or antigenic peptides (Fig. 74.2) (Foster *et al.*, 2003; Szmania *et al.*, 2001). Since the recipients of adoptively transferred T-cells are profoundly immunosuppressed, the use of live/attenuated virus is unwise, but replication incompetent viral vectors expressing a transgene may offer the ideal antigen source, capable of stimulating both CD4+ and CD8+ T cell simultaneously.

APC

The choice of APC for activation of an immune response may differ from that chosen to expand cells to the numbers necessary for infusion and GMP testing. In the case of EBV, LCLs provide an excellent APC with which to stimulate and expand polyclonal EBV-specific T-cells. However, when one considers the treatment of other EBV-associated malignancies such as Hodgkin's disease or NPC where gene expression is limited to the sub-dominant T-cell target antigens LMP1 (Gottschalk *et al.*, 2003), LMP2, and EBNA1, the use of LCLs is problematic since reactivity to early lytic cycle antigens and the immunodominant EBNA3 proteins is preferentially expanded (Rooney *et al.*, 2002; Gottschalk *et al.*, 2003; Rooney *et al.*, 1998, 2001). Thus, transducing LCLs, activated monocytes, or DCs with a viral vector which over-expresses the tumor-associated antigens may be advantageous for the simulation and expansion of a CTL line with more focused reactivity (Keever-Taylor *et al.*, 2001; Leen *et al.*, 2006a). In the case of CMV, where there is a distinct lack of an ideal APC, a number of groups have relied on the generation of DCs, which are then pulsed with CMV lysate or epitope peptide (Szmania *et al.*, 2001), or transduced with vectors expressing immunodominant T-cell antigens (Bonini *et al.*, 2001; Keever-Taylor *et al.*, 2001), as a means to stimulate reactivity (Fig. 74.2). However, although DCs are the most potent APCs available, their inability to

proliferate in vitro means that large blood volumes are required to produce sufficient DC for CTL expansion, therefore DC numbers are limiting, and DC generation is time consuming.

Selection of specific T-cells

More recently, tetramer technology has facilitated the isolation of antigen-specific T-cells directly from peripheral blood (Fig. 74.2). Tetramers consist of four biotin-labeled HLA molecules bound together with streptavidin (Altman *et al.*, 2001) and labeled with fluorochrome. Each HLA molecule is associated with a peptide epitope that binds to peptide-specific CTLs, which can then be directly visualized and isolated by fluorescence-activated cell-sorting (FACS). Once isolated, these cells can either be infused immediately or expanded ex vivo. This method precludes the carry-over of contaminating alloreactive T-cells, which could potentially induce GvHD in vivo. Cobbold and colleagues have recently used this approach to select CMV-specific T cells for the treatment of HSCT patients. CMV-specific $CD8^+$ T cells were isolated from the blood of stem cell transplant donors using HLA-peptide tetramers followed by selection with magnetic beads. The selected cells were infused directly into nine patients within 4 hours of selection. The cell dose was lower than those infused in conventional immunotherapeutic strategies (medium cell dosage was $8.6 \times 10(3)$/kg), however CMV-specific $CD8^+$ T cells became detectable in all patients within 10 days of infusion, and TCR clonotype analysis showed persistence of infused cells in two patients studied. CMV viremia was reduced in every case and eight patients cleared the infection, including one patient who had a prolonged history of CMV infection that was refractory to antiviral therapy. This novel approach to adoptive transfer has considerable potential for antigen-specific T cell therapy (Cobbold *et al.*, 2005). However, each tetramer is specific for only one epitope limiting the repertoire of infused T-cells and, as yet, there are limited HLA class II tetramers available. Thus infused CTL lines may not persist in vivo due to lack of CD4+ help, and can be made only for patients with specific HLA phenotypes.

CTL expansion

The ideal immunotherapy protocol requires the generation of polyclonal virus-specific CD4+ and CD8+ T-cells in the shortest time possible. As cells are activated they begin to expand and through subsequent rounds of expansion, more and more APCs are needed for restimulation purposes. In particular, the need to expand large numbers of virus-specific CTLs for infusion post-SOT is a persistent problem. The number of APCs required for such protocols is frequently a limiting factor. Vaz-Santiago and colleagues tried to circumvent this problem by using PBMCs pulsed with a soluble recombinant chimeric protein, IE1-pp65, to activate and expand memory CD4+ and CD8+ CMV-specific T-cells but while CMV-specific T-cells could be activated, the problems associated with the expansion of sufficient numbers of cells for infusion and testing purposes were not dealt with in this study (Vaz-Santiago *et al.*, 2002). Sili and colleagues have addressed this issue by utilizing Ad5f35pp65-transduced DCs to activate specific T-cells and then successfully expanded the stimulated population using autologous LCLs transduced with the Ad5f35pp65 vector, without loss of CMV specificity (Sili *et al.*, 2003). Alternative expansion programs rely on the use of activated B and T cells, or the use of artificial APCs (aAPCs) (Melenhorst *et al.*, 2006; Coughlin *et al.*, 2004; Mans *et al.*, 2003), but to date success has been limited by the loss of antigen specificity over time. An emerging option for both the activation and the expansion of virus-specific T-cells from a mixed PBMC population lies with the development of novel aAPCs based on the use of an antigen-HLA-epitope peptide complex coupled with a co-stimulatory molecule. Oelke and colleagues have recently pioneered such an approach by immobilizing a dimeric HLA immunoglobulin (HLA-Ig) onto beads together with CD28-specific antibody; the HLA molecules is then loaded with a known epitope peptide and used to activate and expand antigen-specific T cells from peripheral blood (Oelke *et al.*, 2003). Resultant CTL lines were compared with those attained by conventional DC stimulation, and aAPC-based CTL activation was found to be equivalent or even better than DC-induced activation, with no appreciable loss in antigen specificity or growth rate detected over time. Thus, future immunotherapy trials may adopt such HLA-based aAPCs as being more cost effective than current methods of CTL generation and expansion. In addition, aAPCs can be prepared in bulk in advance and stored for use at a later date without loss of function, thus a more standardized approach to the generation of CTL lines for immunotherapy purposes is available. However, this method is limited by the requirement for specific peptides for folding into the HLA molecule, which also restricts the breadth of the CTL repertoire.

Regulation

The final barrier to initiating an adoptive immunotherapy trial for the prophylaxis and/or treatment of herpesvirus infections in the human host relates to the extensive

regulations and time consuming applications which must be submitted to a number of regulatory agencies including the Internal Review Board (IRB), Recombinant DNA Advisory committee (RAC), and Food and Drug Administration (FDA) prior to initiation of any novel immunotherapy protocols. Few Institutions have the infrastructure and facilities required to fulfill all of these regulatory requirements. In addition, each reagent needed for the generation of a CTL line must be available as a clinical grade product, necessitating extensive and expensive analysis and testing. To date, for example, not all the components needed for the generation of mature monocyte-derived DCs are obtainable as clinical grade products due to the high costs of quality assurance and control costs which must be undertaken before a product is certified for human use.

Conclusions and future considerations

Herpesviruses are a significant cause of morbidity and mortality in immunocompromised individuals. Adoptive transfer of virus-specific CTLs has proven safe and effective at preventing and treating such infections in a number of instances, but there is room for improvement in the activation and expansion protocols utilized to allow the generation of virus-specific CTL lines in the shortest time possible. The major criticism of many immunotherapeutic approaches discussed is that they rely on the presence of a seropositive donor, from which specific T-cells can be activated and expanded. Thus, extension of this work to include seronegative SOT recipients or stem cell donors will increase the scope of this treatment to include all patients at risk of developing an infection or having a viral reactivation post-transplant (Savoldo *et al.*, 2002; Park *et al.*, 2006). New and more sensitive techniques to accurately monitor viral loads and thus predict viral reactivations in vivo have also been developed and used to predict EBV and CMV infections in immunocompromised individuals, allowing pre-emptive treatment, and monitoring of the efficacy post-treatment (Rooney *et al.*, 1995a; Wagner *et al.*, 2003b). In the future such monitoring may be extended to cover all herpesviruses, thereby utilizing a rational approach to the treatment of resultant infections either using adoptive immunotherapy or conventional antiviral reagents.

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Immunotherapy of HSV infections – antibody delivery

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Passive immunization involves utilizing polyclonal or monoclonal antibodies as a form of immunotherapy. Antibodies can mediate their effects through several mechanisms, including opsonization and C-mediated lysis, but in particular antibody-dependent cell-mediated cytolysis (ADCC) and neutralization. Antibody immunotherapy has been demonstrated to be efficacious for the treatment and prevention of infection or disease caused by viruses other than herpes simplex virus (HSV) (Abzug *et al.*, 1995; Reed *et al.*, 1988; Feltes *et al.*, 2003; Saez-Llorens *et al.*, 1998; Subramanian *et al.*, 1998; The IMpact-RSV Study Group, 1998). While intriguing data exist in animal models suggesting that such a therapeutic intervention may also be of benefit in the management of HSV infections, to date no controlled studies have demonstrated the benefit of such an approach in humans. This chapter explores the potential of such an approach in people, as well as the limitations in current knowledge.

Immune responses following HSV infection

Host resistance to HSV infections includes non-specific mechanisms such as interferons, neutrophils, complement, macrophages, and natural killer cells, as well as specific mechanisms including humoral (antibody) immunity, T-cell-mediated immunity (such as cytotoxic T-cells and T-helper activity), and cytokine release. The relative importance of these various mechanisms is different for initial vs. recurrent HSV disease. Animal studies suggest that activated macrophages, interferons, and, to a lesser extent, natural killer cells are important in limiting initial HSV infection, whereas humoral immunity and cell-mediated immunity are important in controlling both initial and recurrent infections. Adoptive transfer studies suggest that either virus-specific antibody or lymphocytes can protect animals against initial HSV infection (as discussed below), but several lines of evidence suggest that cell mediated immunity responses play the central role in controlling recurrent HSV infections (Koelle *et al.*, 2000; Posavad *et al.*, 1996, 1997, 2000; Stanberry *et al.*, 2000). Mucocutaneous herpes is more severe in patients with impaired or defective cell mediated immunity (Posavad *et al.*, 1997; Whitley *et al.*, 1998), but not in patients with agammaglobulinemia.

Following acquisition of HSV-1 or HSV-2, IgM antibodies appear transiently and are followed by production of IgG and IgA antibodies which persist over time. Both neutralizing antibodies and antibody-dependent cellular cytotoxic (ADCC) antibodies generally appear between 2 weeks and 6 weeks following infection and persist for the lifetime of the host. The host response to virus-specific infected cell polypeptides and the development of neutralizing antibodies have been defined through immunoblot and immunoprecipitation assays (Bernstein *et al.*, 1985; Eberle *et al.*, 1981). Following infection, antibodies directed against glycoprotein (g)B, gC, gD, gE, gG1, gG2, and ICP-4 appear sequentially. Of note, the intensity of host antibody responses to virus-specific polypeptides and high concentrations of neutralizing antibodies are not protective against HSV recurrences.

Neonates

The host responses of neonates with HSV disease differ from those of older children and adults. Infected neonates will produce HSV-specific IgM antibodies (as detected by immunofluorescence) within three weeks of acquisition of the viral infection. HSV-specific IgM concentrations increase rapidly during the first 2 to 3 months, and in some infants may be detectable for as long as one year following

Table 75.1. Protective effect of neutralizing antibody in prevention of neonatal HSV disease

Neutralizing antibody titer (cord blood or 2 weeks of life)	Exposed during delivery but uninfected (n=33)	Exposed during delivery and infected (n=29)
<1:5	0 (0%)	12 (41%) $P<0.00001$
1:5 to 1:20	7 (21%)	15 (52%)
>1:20	26 (79%)	2 (7%) $P<0.0002$

From Prober *et al.* (1987).

neonatal infection. HSV IgG antibodies also appear by 3–4 weeks in most infants. The viral surface glycoproteins gB and gD are the most reactive immunodeterminants (Sullender *et al.*, 1987) and, indeed, account for the majority of neutralizing antibodies.

Human studies suggesting of protection by HSV antibodies

Adults

Type-specific antibodies against one type of HSV may have limited ability to protect against acquisition of the other HSV type. For example, pre-existing antibody directed against HSV-1 correlates with protection against acquisition of genital HSV-2 infection (Breinig *et al.*, 1990; Cowan *et al.*, 1994; Eberhart-Phillips *et al.*, 1998; Fleming *et al.*, 1997; Gibson *et al.*, 1990; Johnson *et al.*, 1989; Mertz *et al.*, 1992; Nahmias *et al.*, 1990; Rosenthal *et al.*, 1997). The influence of pre-existing type-specific HSV-1 antibodies on acquisition of HSV-2 infection can also be inferred from recent successes, although somewhat limited, in HSV vaccine development. A candidate HSV-2 glycoprotein D subunit vaccine adjuvanted with alum combined with 3-deacylated monophosphoryl lipid A (MPL) has recently demonstrated promising results. In two large Phase III studies, the vaccine has been demonstrated to be safe and, in a subset of volunteers, effective in preventing HSV-1 or -2 genital herpes disease (vaccine efficacy ~75%) and HSV-2 infection (vaccine efficacy ~40%) (Stanberry *et al.*, 2002). In both studies, efficacy was limited to women who were HSV-1 and -2 seronegative prior to vaccination. There was no evidence of vaccine efficacy in men or in women who were HSV 1+/2- prior to vaccination.

While there is some degree of cross-protection conferred by pre-existing HSV-1 antibody on the acquisition of HSV-2 infection, the protection is incomplete. Similarly, vaccines have been developed which generate robust humoral responses yet fail to protect against HSV-2 infection (Stanberry *et al.*, 2000). As such, it is difficult to envision a circumstance whereby passive antibody immunotherapy will play a role in the management of genital HSV infection and disease.

Neonates

Both maternal antibody status (Brown *et al.*, 2003; Prober *et al.*, 1987; Yeager and Arvin, 1984; Yeager *et al.*, 1980) and type of maternal infection (primary vs. recurrent) (Brown *et al.*, 1987, 1991, 2003; Corey and Wald, 1999; Nahmias *et al.*, 1971) influence transmission of HSV from mother to baby. Neonates with higher neutralizing antibody titers acquired transplacentally are less likely to become infected with HSV following perinatal exposure of passage through an infected birth canal (Prober *et al.*, 1987), illustrating the protective effects of preexisting antibody in preventing neonatal HSV disease (Table 75.1). Additionally, infants born to mothers who have a first episode of genital HSV infection near term are at much greater risk of developing neonatal herpes than are those whose mothers have recurrent genital herpes (Brown *et al.*, 1987, 1991, 2003; Corey and Wald, 1999; Nahmias *et al.*, 1971). This increased risk, however, is not solely due to lower concentrations of transplacentally passaged HSV-specific antibody, since women with first episode disease also shed virus for a longer period of time and in higher quantities. The largest assessment of the influence of type of maternal infection on likelihood of neonatal transmission involved almost 40,000 women without clinical evidence of genital HSV infection who were cultured within 48 hours of delivery (Fig. 75.1). Of these, 121 women were identified who both were asymptomatically shedding HSV and for whom sera were available for serologic analysis. In this large trial, 57% of babies delivered to women with first episode primary infection developed neonatal HSV disease, compared with 25% of babies delivered to women with first episode non-primary

Table 75.2. Protective effect of neutralizing antibody in limiting extent of neonatal HSV disease

Neutralizing antibody titer (1 week of life)	Extent of disease		
	SEM (n=17)	CNS (n=19)	Disseminated (n=11)
<1:5	6 (35%)	2 (11%)	8 (73%) P<0.01
1:5 to 1:20	8 (47%)	15 (78%)	3 (27%)
>1:20	3 (18%)	2 (11%)	0 (0%)

From Sullender *et al.* (1987).

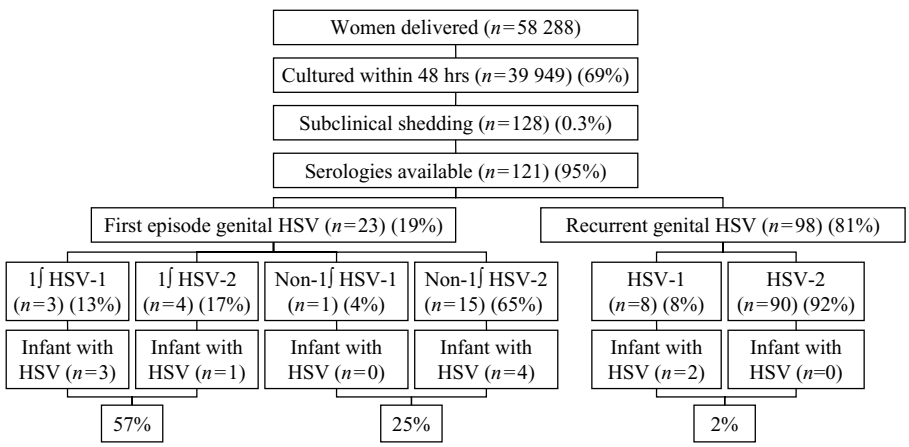

Fig. 75.1. Type of maternal infection and risk of HSV transmission to the neonate. (Data from Brown *et al.*, 2003.)

infection and 2% of babies delivered to women with recurrent HSV disease (Brown *et al.*, 2003).

Among HSV-infected neonates, anti-HSV neutralizing antibody titers have been shown to correlate with the extent of the disease (Sullender *et al.*, 1987), with babies with higher neutralizing antibody titers being more likely to have localized disease (and less likely to have disseminated disease) once they are infected (Table 75.2). Similarly, high maternal or neonatal anti-HSV ADCC antibody levels or high neonatal antiviral neutralizing levels are each independently associated with an absence of disseminated HSV infection (Kohl *et al.*, 1989) (Table 75.3).

Animal models of antibody immunotherapy in HSV infection and disease

The natural immune responses to HSV infection, both humoral and cellular, are strongly directed against the surface glycoproteins gB and gD, and both human and humanized antibodies directed against gB and gD have been shown to be beneficial as prophylactic and therapeutic agents in animal models of HSV infection (Baron *et al.*, 1976; Bravo *et al.*, 1996; Kern *et al.*, 1992; Lake *et al.*, 1992). In models of disease prevention, administration of polyclonal or monoclonal neutralizing antibodies prior to infection with HSV confers significant protection in mice. Similarly, administration of antibodies as late as 72 hours after infection dramatically decreases mortality as well as the quantity of virus detected in organs such as the brain and lungs (Kern *et al.*, 1992). In a murine model of neonatal HSV disease with a high challenge dose, protection was highly associated with ADCC activity (Kohl *et al.*, 1990). In a low dose challenge model, neutralizing activity of antibody alone was associated with protection in vivo (Kohl *et al.*, 1990). In a guinea pig model of neonatal HSV, combination therapy with passive anti-HSV antibody and acyclovir was effective even when administered on day 3 post-infection, reducing mortality from 82% to 44%. Acyclovir alone was effective only when begun on day 0, and antibody alone

Table 75.3. Protective effect of neutralizing and ADCC antibody in limiting extent of neonatal HSV disease

	Neonatal neutralizing antibody titer		
Neonatal ADCC	<1:5 (n=15)	1:5 to 1:20 (n=25)	>1:20 (n=6)
SEM			
$1:10^4$ to $1:10^6$	2	6	3
0 to $1:10^3$	4	2	2
CNS			
$1:10^4$ to $1:10^6$	2	5	0
0 to $1:10^3$	0	9	0
Disseminated			
$1:10^4$ to $1:10^6$	0	0	0
0 to $1:10^3$	7	3	1

From Kohl *et al.* (1989).

was effective when begun on or before day 2 (Bravo *et al.*, 1996). Also in guinea pigs, administration of HSV-specific antibody protects the sacral ganglia from HSV infection resulting from vaginal challenge (Bourne and Stanberry, 1993), suggesting a role for antibody in protecting the neuron early in primary HSV infection, possibly by blocking infection of the sensory nerve ending or alternatively by acting at the level of the ganglia (Stanberry *et al.*, 2000).

Limitations of antibody immunotherapy

As noted above, prospects for passive antibody immunotherapy for genital HSV infection and disease seem remote. However, the protection against infection and amelioration of disease severity afforded by neutralizing and ADCC antibodies in neonatal HSV may portend a future role for this manifestation of HSV infection. While antibody therapy offers promise for improving neonatal HSV disease prevention and outcome, studies in humans have yet to be performed. In addition, an HSV hyperimmune globulin preparation does not exist, and the amount of anti-HSV antibodies present in conventional intravenous gammaglobulin (IVIG) preparations is low and variable, such that unacceptable large volumes would need to be injected to potentially confer protective immunity. For these reasons, use of IVIG in the management of neonates with HSV disease cannot be recommended at this time.

The development of human and humanized monoclonal antibodies obviates the current problems with pooled IVIG preparations, and may allow for the systematic evaluation of the therapeutic benefit of passive immunization in neonatal HSV disease. Human monoclonal antibodies offer further potential advantages over murine and chimeric antibodies such as longer circulating half life and reduced or possibly undetectable immunogenicity. At least two human monoclonal antibodies exist which could one day be evaluated in neonatal HSV disease.

HSV 863 is an HSV gD group Ib human monoclonal antibody of IgG1gamma isotype. In vitro studies showed that HSV 863 reacts with all of the 99 strains of HSV-1 and HSV-2 tested. It has potent neutralizing activity in the absence of complement, with an IC_{50} range of 0.05–0.35 μg/ml, with IVIG being approximately 128 to 256 times less potent. The neutralization IC_{50} for HSV 863 in the presence of complement was identical. HSV 863 confers a marked dose-dependent increase in ADCC activity, with maximal ADCC efficacy being achieved with concentrations of HSV 863 as low as 1 μg/ml with HSV-1 and 5 μg/ml with HSV-2. The half life of this monoclonal antibody is approximately 19 days in monkeys, with no apparent side effects, toxicity or immunogenicity following single or multiple dose administration. In vivo, using a neonatal HSV model, HSV 863 proved effective both prophylactically and therapeutically. When administered prior to virus challenge, the ED_{50} was 1–8 mg/kg for protection against a $10 \times LD_{50}$ challenge with HSV-1 or HSV-2. The antibody was protective in dose of 30 mg/kg up to 2 days after viral challenge and up to 72 hours post-infection when administered in dose of 90 mg/kg. In combination treatment with acyclovir, there were indications of additive effects, but no interference. HSV 863 at 10 mg/kg given 72 hours after infection was 90% protective, and doses of 1.5 to 15 mg/kg reduced both skin lesion severity and abundance in a murine model of cutaneous HSV disease. In a murine ascending myelitis model, treatment with HSV 863 or acyclovir substantially reduced the extent of detectable latent ganglionic infection, especially when given prophylactically 24 hours prior to HSV inoculation. Production of this product, though, is currently on hold pending discussions with potential manufacturers of commercial-grade product.

HX-8 is a human monoclonal antibody which binds HSV-1 and -2. It is produced by Epicyte Pharmaceutical in genetically modified corn and neutralizes both HSV-1 and HSV-2. It is currently being developed as a topical human antibody, but to date has not been tested in humans.

Summary

The immunobiology of HSV infections is complex. Cell mediated and humoral immunities are both important in the immunologic response to HSV infection, but millennia

of evolution have provided the virus with the means by which to evade even the intact immune system, both maintaining latency and allowing for intermittent reactivation of disease. These facts likely limit the utility of passive antibody immunotherapy in the management of genital HSV infection. However, evidence for protection against infection and amelioration of disease severity afforded by neutralizing and ADCC antibodies in neonatal HSV may portend a future role for this manifestation of HSV infection. While antibody therapy offers promise for improving neonatal HSV disease prevention and outcome, studies in humans have yet to be performed.

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Part VIII

Herpesviruses as Therapeutic Agents

Edited by Richard Whitley and Bernard Roizman

76

Herpesviruses as therapeutic agents

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Introduction

After more than a decade of intensive research and development efforts, the translation of promising viral-based gene therapies from the research lab to the clinic is both promising and unexpectedly challenging. Many of the same properties that make viral vectors attractive candidates to deliver genes for therapeutic purposes also impede the path to successful clinical development.

Vectors for clinical use must be manufactured in relatively high yields such that hundreds of thousands or even millions of "doses" can be generated in a safe and cost-effective manner. Moreover, the resulting vector must exhibit genetic as well as structural stability, withstand storage at various temperatures for up to several years, and cause little or no toxicity in animals, and ultimately in humans.

Herpes simplex viruses (HSV), while widespread in nature, have not been tested in human clinical studies as often as several other commonly used vectors, such as adenovirus, adeno-associated viruses (AAV), and retroviruses. In many ways however, HSV is emerging as a viable therapeutic platform and several clinical studies are either ongoing or planned for the very near future. The reason for this increased focus on HSV is due in part attributable to the unique properties that make HSV a stable and potentially potent vector for controlled gene delivery. In addition, the increasing experience with replication-competent vectors in human clinical studies has made it more familiar with clinicians.

Properties of therapeutic HSV vectors

Therapeutic HSV can be characterized as replication-competent or replication-defective. Replication competent vectors, such as the oncolytic vectors described in this chapter, are usually deleted for "non-essential" viral genes to render the vector less "toxic" as measured by the ability to cause disease in susceptible animal models. These vectors are often referred to as "replication-attenuated." If they are designed to treat cancer, they are "oncolytic" in the sense that they are at least partially replication competent in tumor cells, and largely replication defective in surrounding tissue.

This differential permissiveness has been shown in several studies to be due to the unique environment of the tumor cell vs. the "normal" surrounding cell. For example, HSV-1 mutants such as G207 are now being tested in clinical trials for anti-cancer activity. R3616, which is similar to G207, is an HSV-1 mutant that is deleted for the viral anti-PKR gene, $\gamma_1$34.5. The R3616 mutant infects *ras*-transformed but not untransformed cells, which suggests that the Ras-signaling pathway compensates for the loss of the virus' own anti-PKR mechanism (Farassati *et al.*, 2001). HSV-1, therefore, predominantly uses the host anti-PKR mechanism to infect transformed cells and its selectiveness for transformed cells makes it a good candidate for an anti-cancer therapeutic.

Replication defective HSV vectors are deleted for one or more genes that are known to be essential for viral replication in otherwise permissive cells. As a result, defective vectors can only be manufactured using cell lines that are modified to contain the complementing viral genes. These vectors often exhibit reduced toxicity in non-complementing cells compared to wild-type virus, and appear to be largely non-toxic when administered to animals. Although many different replication defective HSV vectors have been generated, only a few of them represent viable candidates for clinical development, and none have been tested in humans to date.

Properties of non-replicating HSV vectors for therapeutic use

There are two basic approaches to generating replication defective HSV vectors: amplicons and whole genome vectors.

Plasmid-based amplicon vectors

Amplicons, first described over 20 years ago (Spaete and Frenkel, 1982), are defective HSV particles produced from plasmids containing the gene of interest, an origin of replication and a packaging signal. Plasmids are incapable of replicating without HSV helper functions, either in the form of helper virus, or resident on cosmids or bacterial artificial chromosomes. The resulting vectors contain multiple copies of the therapeutic gene, and are capable of infecting cells via the HSV entry pathway. Despite the potential safety advantages of such a system, in practice it is relatively difficult to generate high titer stocks devoid of helper virus. Although advances in generating helper-free stocks have helped somewhat (Fraefel *et al.*, 1996), this is offset at least in part by an increase in the complexity of manufacturing. Moreover, the plasmid sequences transferred into cells by amplicons are not HSV genomes, so consistent, regulated expression has been difficult to achieve in animals. Amplicon vectors are unique in their ability to deliver up to 153 kb of heterologous DNA into a wide range of mammalian cells (Wade-Martins *et al.*, 2001).

In an attempt to extend the stability and duration of expression from amplicon vectors, and thus improve potential usefulness in gene therapy, hybrid amplicons consisting of elements of the AAV genome were constructed and tested for their ability to direct stable gene transduction by site-specific integration (Wang *et al.*, 2002). The vector constructs, which contained the AAV inverted terminal repeats and the Rep gene were packaged as amplicons and compared to a standard amplicon vector in cell culture. Interestingly, the hybrid vectors improved the stable transduction frequency compared with controls, and the transgene was integrated into the host cell chromosome. The ability of the HSV amplicon construct to integrate into the host cell chromosome may hold promise for further improvements in long-term gene expression in situations where long-term gene expression is a priority.

Another approach for improving the efficiency of HSV-derived amplicon vectors has been to re-target the amplicon to make it more cell-type specific by reducing its ability to attach to all but the target cells. Grandi *et al.* (2004) have developed a method to re-target amplicon vectors by replacing the gC heparan sulfate binding domain with a model ligand, in this instance the hexameric histidine tag. They demonstrated enhanced binding of modified virus to receptor-positive cells with no loss of infectivity. This approach holds promise for reducing the dose required for adequate therapeutic effect. This would also have the potential to improve the prospect for the manufacture of clinical supplies. These and other enhancements will first require optimization in animal models prior to their inclusion in HSV therapeutics intended for human use.

Replication defective whole genome vectors

The second vector type is represented by HSV genomes deleted for essential genes. To grow these viruses, cell lines approved for clinical manufacturing are modified to contain the viral gene or genes required to complement the defect in the defective vector. Deletion of one or other of the essential immediate-early genes (ICP4, ICP27) results in a virus that cannot replicate (DeLuca *et al.*, 1985; McCarthy *et al.*, 1989; Samaniego *et al.*, 1997, 1998; Wu *et al.*, 1996), except in cells that complement the null mutations by providing ICP4 or ICP27 in trans (DeLuca *et al.*, 1985; Samaniego *et al.*, 1998; Wu *et al.*, 1996). In practice, the only potentially clinically useful vector strains have two or more deletions to reduce the potential that a recombination event with the viral gene resident in the complementing cell will lead to a replication competent contaminant during manufacturing. Multiply deleted vectors cannot replicate in non-complementing cells and do not express early or late genes. These vectors can also contain additional defects in viral genes that lead to desirable properties such as increased yield, reduced cytotoxicity, genome stability or altered immune response.

Another approach for increasing the yield of non-replicating vectors is by modifying the complementing cells. For example, complementing cell lines can be made to express the complementing sequences only upon HSV infection, thereby reducing cytotoxicity and enabling robust replication and high yields during manufacture. Viruses generated in this manner can be designed to be relatively non-toxic, especially in neuronally- derived cell types (Palmer *et al.*, 2000; Lilley *et al.*, 2001).

Use of HSV vectors to modify the nervous system

Herpes simplex virus vectors are particularly well suited for the delivery of genes to neurons. In natural infections, the wild-type virus particle targets with high efficiency from peripheral inoculation to the nucleus of sensory neurons in the dorsal root ganglion. Once in the nucleus, the viral

genomes establish latency, in which they persist for the life of the host as intranuclear episomal elements. Although wild-type virus may be reactivated from latency, recombinant vectors that are entirely replication defective retain the ability to establish a persistent state in neurons, but are unable to replicate (or reactivate) in the nervous system.

Most viral gene expression is attenuated as the virus enters latency and is tightly regulated. Fortunately, the virus does express the LAT transcripts, which indicates that there is no absolute block to HSV-based gene expression in the neuron following the establishment of latency. Chimeric promoter constructs containing the LAT promoter enhancer region can confer long-term activity on otherwise silenced exogenous promoters (Palmer *et al.*, 2000; Lilley *et al.*, 2001). Vectors in which the LAT promoter is made to control exogenous gene expression have also been shown to express foreign genes in mouse dorsal root ganglia (DRG) for months following footpad inoculation (Marshall *et al.*, 2000). These vectors allow long term expression in dorsal root ganglia and the CNS of animal models. They also retain the ability to be transported via the axon to distant sites, a feature that may be useful for targeted delivery to the peripheral or central nervous system in the clinical setting.

Peripheral nervous system (PNS)

Latency of wild-type HSV occurs in the sensory ganglia of peripheral nerves and long-term expression of transgenes from HSV vector has proved feasible in the PNS (Goins *et al.*, 1999). Expression of NGF and other neurotrophic factors using the LAP2 and heterologous promoters to drive expression has persisted long enough to demonstrate the therapeutic potential of such constructs in the clinical setting for the treatment of neuropathy (Chattopadhyay *et al.*, 2002, 2003; Goss *et al.*, 2002a,b). Expression of enkephalin peptides from defective HSV vector backbones has also been demonstrated in animal models of chronic pain suggesting that as little as several weeks of gene expression might provide therapeutic benefit (Goss *et al.*, 2001, 2002a, b; Hao *et al.*, 2003).

Central nervous system (CNS)

It would normally be expected that infection of the CNS with HSV would cause encephalitis, by contrast with the latent infection established in the PNS. It has been demonstrated quite convincingly, however, that even replication competent HSV constructs such as the oncolytic vectors G207 and strain 1716 can be delivered safely to the human brain for the treatment of malignant glioma. Therefore it would be expected that replication defective HSV should be non-toxic following CNS delivery. In fact, this has been demonstrated in animals for certain HSV recombinants with multiple IE gene deletions (Krisky *et al.*, 1998), or with IE gene deletions (ICP4, 27) coupled with inactivating mutations in ICP34.5/open-reading frame P and VP16 (Lilley *et al.*, 2001). Vectors such as these would be highly appropriate vehicles to arm with therapeutic genes either to preserve or modify neuronal function or to destroy tumor cells. These vectors express minimal levels of any of the IE genes in non-complementing cells and transgene expression can be maintained for extended periods with promoter systems containing LAT promoter elements (Palmer *et al.*, 2000).

Additionally, HSV-based vectors allow highly effective gene delivery both to cultured neurons and to the CNS in animal models. Moreover, these vectors are efficiently transported from the site of inoculation to connected sites within the CNS and less so within the PNS. This has been demonstrated by gene delivery to both the striatum and substantia nigra following striatal inoculation, to the spinal cord, spinal ganglia, and brainstem following spinal cord infection, and to retinal ganglia neurons following injection in the superior colliculus and thalamus (Lilley *et al.*, 2001).

In a comprehensive study of vector delivery, Palmer *et al.* (2000) have identified combinations of deletions from the HSV genome that allow efficient gene delivery to spinal dorsal root ganglia (DRGs) following peripheral inoculation into the footpad or sciatic nerve of mice. This showed that LAT promoter elements can confer long term expression in the context of different exogenous promoters, and that partial replication-competence may be more efficient for gene delivery following footpad or nerve injection. The efficiency of retrograde transport and subsequent gene expression following footpad or sciatic nerve inoculation was determined to be dose dependent, and not as efficient as with replication competent vectors. This may be due to the need to penetrate a barrier of accessory cells or otherwise increase the relative amount of vector available at the site of inoculation.

These unique viruses show an encouraging lack of toxicity following injection as well as relatively long-term expression: capabilities that have important implications for therapeutic uses where target neurons are in regions of the nervous system that are relatively inaccessible by normal therapeutic techniques. These target cells might include the dopaminergic neurons of the substantia nigra that are affected in Parkinson's disease (Yamada *et al.*, 1999), the retinal ganglion cells, or delivery to the DRG for the treatment of pain, or to repair the spinal cord or nerves.

Malignant glioma

Malignant glioma is a malignancy of the CNS that can be treated by surgery, radiation, and chemotherapy. To date, only replication competent HSV has been tested in clinical studies with some success to treat refractory disease. Replication defective vector candidates have also been designed to arm the HSV vector in such a way to provide a more potent antitumor effect than has been demonstrated so far with the replication-attenuated vectors.

Interestingly, the TK gene of HSV has been introduced into a variety of non-HSV vectors and tested for its ability to kill tumor cells in the presence of ganciclovir, but never in the HSV vector itself. Candidate HSV vectors have been generated that are equipped with additional genes that might be useful to treat brain tumors. For example, connexin 43, which is the major component of astrocyte gap junctions, has been introduced into an HSV vector to increase the formation of intercellular junctions in a manner that may potentiate HSV/tk-based cancer treatment by promoting the transfer of activated ganciclovir from cell to cell (Sanson *et al.*, 2002). Likewise, inclusion of antitumor factors such as the cytokine TNF alpha appear to provide added benefit if expressed in high concentrations in the tumor and surrounding tissue from an HSV vector backbone (Moriuchi *et al.*, 1998).

Additional work has been performed in an attempt to regulate vector-directed gene expression such that it is either naturally activated in the tumor cell environment or induced at will through the use of exogenous inducers. In this regard, HSV-1 amplicon vectors that are regulated by the cell cycle have been developed to generate glioma-specific HSV amplicon therapeutics (Ho *et al.*, 2004). The design of one such construct is based on the observation that cell cycle dependent factor CDF-1 appears to be specifically expressed during the G(0)/G(1) phase of the cell cycle and its binding site is located within the cyclin A promoter. In non-dividing cells, transactivation of a cyclin A promoter via the interaction of a Gal4/NF-YA fusion protein with Gal4-binding sites is prevented by the presence of CDF-1, which acts as a repressor. By contrast, in proliferating cells such as those in actively growing tumor, CDF-1 is presumably absent and transactivation occurs. These regulatory elements have been incorporated, along with tissue-specific elements such as the GFAP enhancer, into HSV amplicon vectors to target expression to a specific group of cells. This construct has been shown to exhibit both cell-type specific and cell cycle dependent transgene expression in glioma-bearing animals. These vector properties, if active in animal models of disease, would reduce the need for targeting at the level of cell attachment and entry, and may represent an important step in developing a vector that can be delivered systemically or locoregionally to target the maximum number of tumor cells in the context of metastatic disease.

Another approach to gene regulation adapted to the HSV system that is not limited to glioma therapy, is the tetracycline-inducible gene expression system. Of the known eukaryotic regulatory systems, the tet-inducible system is perhaps the most widely used because of its tight regulation and the availability of tetracycline, which is suitable for human clinical use. These constructs contain a tetracycline response element (TRE) linked to a promoter such as the heterologous promoter CMV, or the HSV ICP0 promoter (Schmeisser *et al.*, 2002). Studies have demonstrated that these constructs result in tetracycline inducible gene expression that is not cell type specific, and are capable of inducible expression for several days in irreversibly differentiated NT2 cells, which is a neuronally committed human teratocarcinoma cell line that differentiates into neuron-like hNT cells following treatment with retinoic acid. The ability to turn gene expression on and off following intratumoral or systemic delivery of an HSV-based cancer therapeutic could allow for the inclusion of increasingly potent and potentially toxic gene products into HSV vectors to enhance antitumor efficacy.

Towards optimizing HSV vectors for therapeutic use

One of the most difficult problems in developing safe and efficient viral therapeutics is to determine the optimal dose of vector that will be effective in humans. In an effort to address this problem, the HSV TK gene has been shown to function as a useful marker gene for the direct in vivo localization of TK expression by positron emission tomography (PET). Several double and triple HSV amplicon vector constructs expressing HSV-1 TK, green fluorescent proteins, and *E. coli* cytosine deaminase have been generated and tested by injecting between 10^7 and 10^8 transducing units into subcutaneously Gli36dEGFR gliomas in nude mice. All amplicon vector constructs mediated GFP expression and sensitized the cells toward ganciclovir and 5-fluro-cytosine mediated cell killing in a drug-dose dependent manner (Jacobs *et al.*, 2003.). Moreover, functional proportional coexpression of the PET marker gene TK and the linked therapeutic E. coli CD gene was observed. TK expression could be imaged by PET in vivo even with apparent suboptimal transduction and gene expression. This raises the exciting possibility that the expression of HSV TK from

therapeutic HSV vectors could serve as a useful marker for non-invasive imaging of vector distribution following administration. This would be extremely useful in Phase I and Phase II clinical trials and could provide a powerful means for optimizing dose, formulation, and the rate and mode of delivery.

Non-replicating vectors: current trends

No replication-defective HSV vector has yet been tested in human clinical studies for the treatment of disease. This is somewhat surprising given that replication competent vectors were first introduced into humans in 1998 for the treatment of malignant glioma. It is likely that this situation will change now that the clinical experience with replication competent HSV has been largely positive. Replication defective HSV vectors have many desirable qualities for use as therapeutics. They can be engineered to express several genes at once, manufactured in complementing cell lines that have the potential to yield more replication-defective virus than the replication-attenuated vectors already in use, and they do not replicate following administration to the patient. Thus, the effective dose cannot increase following administration. In this way, replication-defective vectors are more "drug-like" than are the replication competent vectors. The possibility to equip the vector with potentially toxic gene products for cancer therapy or to effect long term gene expression in the nervous system for the treatment of pain, neuropathy or CNS disease will provide the motivation to move these novel therapeutics from the research laboratory to the clinic.

Oncolytic HSV

Introduction

The original genetically engineered viruses examined as antitumor oncolytic agents studied as it was already a well-studied, non-integrating, neurotropic virus with oncolytic were HSV-1 mutants utilized in a preclinical models of human glioma (Markert *et al.*, 1992; Martuza *et al.*, 1991). HSV-1 was properties whose essential and non-essential genes have been identified (Fig. 76.1(a)). Deletion of the neurovirulence genes from an oncolytic HSV vector can effectively produce selective targeting of tumor cells for oncolysis. It has been estimated that 30 kilobase-pairs of the HSV genome could be replaced with foreign DNA while maintaining virus replication. Clinically utilized anti-viral drug regimens already exist to interrupt HSV infection, which adds a margin of safety to its clinical use as an oncolytic agent.

The first engineered replication-competent HSV-1-derivative examined for oncolytic effects was *dl*sptk, a thymidine kinase deletion mutant (Martuza *et al.*, 1991). As a result of this mutation, *dl*sptk can only replicate in mitotically-active cells that can supply both thymidine kinase and pools of available nucleotides necessary for viral DNA replication. Efficacy was demonstrated in multiple animal models of glioma and other nervous system tumors (Martuza *et al.*, 1991; Markert *et al.*, 1993). However, clinical studies were not pursued with *dl*sptk because (i) evidence of low-level encephalitis was seen on histological examination of treated mice, and (ii) its tk mutation made it resistant to, the major antivirals in use for HSV infection, acyclovir and ganciclovir. Other HSV-1 vectors studied with markedly decreased virulence in the normal CNS but capable of oncolysis include R3616 (which contains a one kilobase deletion in both copies of the $\gamma_1$34.5 gene locus) (Chou *et al.*, 1990; Chou and Roizman, 1992)(Fig. 76.1(b)) and hrR3 (which contains a disabling *lacZ* insertion into the U_L39 locus) (Goldstein and Weller, 1988) (Fig. 76.1(c)).

The two $\gamma_1$34.5 gene copies that are deleted in R3616 are located in the inverted repeat regions flanking the Unique Long (U_L) segment of the HSV-1 genome, and encode a 263 amino acid protein with dual functions. First, $\gamma_1$34.5 is responsible for the neurovirulence properties of HSV-1. Second, the $\gamma_1$34.5 gene product (ICP34.5) subverts a major response of the host cell to HSV infection. Upon viral infection, double-stranded RNA is produced, and a stress response occurs in the host cell. Protein kinase R (PKR) is activated, shutting down translation in the infected cell as an antiviral protective mechanism by phosphorylating and inactivating eukaryotic initiation factor-2α (eIF-2α). ICP34.5 recruits protein phosphatase-1a in order to dephosphorylate eIF-2α and allow protein synthesis and viral replication to proceed (He *et al.*, 1997). Studies have demonstrated that HSV-1 mutants lacking functional ICP34.5 activity produce a lytic infection only in cells with defective PKR pathways (Chou *et al.*, 1990; Chou and Roizman, 1992). Over expression of Ras appears to allow $\Delta\gamma_1$34.5 HSV-1 replication in tumor cells due to a defective PKR pathway (Farassati *et al.*, 2001). R3616 produced no neurovirulence or encephalitis but maintained antiglioma activity in nude mouse models (Markert *et al.*, 1993).

U_L39 encodes the large subunit of the enzyme ribonucleotide reductase (ICP6) which HSV utilizes for nucleotide synthesis after infection of post-mitotic cells, such as neurons, which would otherwise not support the HSV

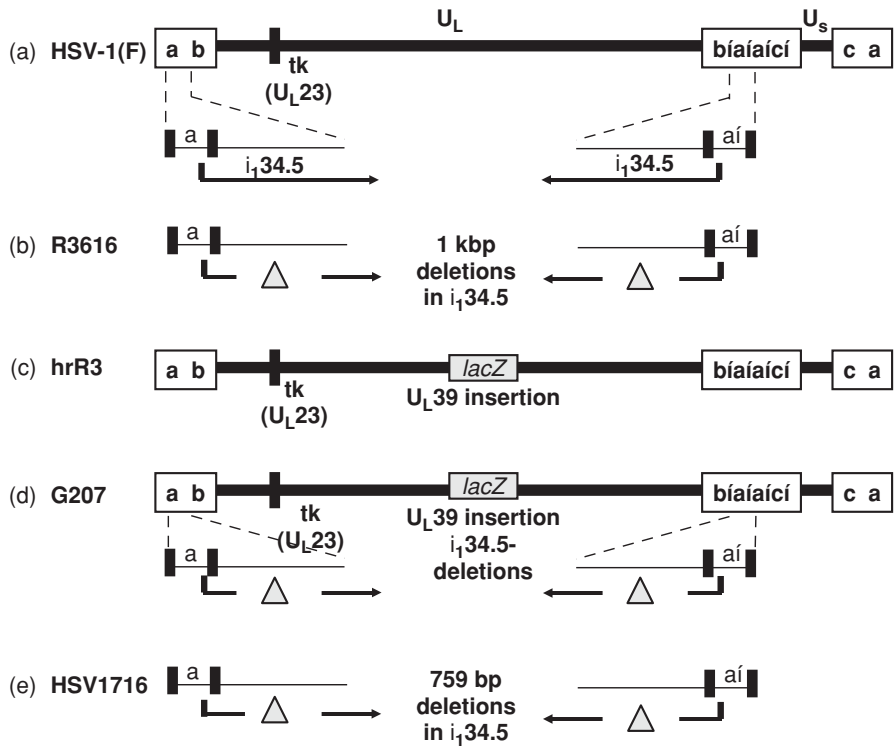

Fig. 76.1. The wild-type herpes simplex virus type 1 genome consists of two major adjacent segments (the Unique long and Unique short segments), each flanked by inverted repeats. The $\gamma_1$34.5 genes are located in the inverted repeats flanking the UL segment. 1b. The R3616 HSV-1 mutant possesses a 1 kilo-basepair deletion in both copies of the $\gamma_1$34.5 gene.1c. hrR3 contains a *lacZ* insertion in the UL39 locus, inactivating the ICP6 (ribonucleotide reductase) gene product.1d. G207 was constructed to possess both the *lacZ* insertion inactivating the UL39 locus and deletions in both genes $\gamma_1$34.5.1e. HSV1716 is similar to R3616 in that too contains deletions in both copies of the $\gamma_1$34.5 gene, inactivating the ICP34.5 protein.(62)

replication cycle. Dividing cells however, can provide cellular ribonucleotide reductase *in trans* obviating the need for the viral protein (Goldstein and Weller, 1988). The oncolytic HSV-1 hrR3 has a *lacZ* insertional mutation in U_L39 renders the viral ribonucleotide reductase nonfunctional. As a result, the virus produces a lytic infection only in dividing cells, including those found in neoplasms, such as malignant glioma (Mineta *et al.*, 1994). A potential additional benefit is that the ribonucleotide reductase-deficient virus is hypersensitive to the anti-viral effects of acyclovir and ganciclovir (Coen *et al.*, 1989)

Oncolytic HSV in malignant glioma

To decrease the possibility of recombination to wild-type virus and further increase the safety of oncolytic HSV-1, a new mutant, G207, was constructed. This virus is a double mutant with deletions in both copies of the $\gamma_1$34.5 gene locus as well as a *lacZ* insertion into the U_L39 locus (Mineta *et al.*, 1995) (Fig. 76.1(d)). G207 retains susceptibility to standard anti-HSV therapies such as acyclovir, since the thymidine kinase gene is intact; due to the inactivation of U_L39, G207 is in fact modestly hypersensitive to acyclovir (Coen *et al.*, 1989; Mineta *et al.*, 1995).

G207 was shown to produce antiglioma effects in multiple preclinical glioma models (Mineta *et al.*, 1995), and was shown to be safe for inoculation at high titers in mice and subsequently in non-human primates (New World owl monkeys *Aotus nancymae*) (Hunter *et al.*, 1999). Of 16 *Aotus*, 13 received intracerebral inoculations of either 1×10^7 or 1×10^9 pfu of G207, two received vehicle and one received 1×10^3 pfu of the wild-type parent HSV-1(F). None of the G207-inoculated animals died due to virus-induced complications (three died from non-neurologic reasons), whereas the control HSV-1(F)-infected monkey succumbed quickly to HSV encephalitis as expected (Hunter *et al.*, 1999). Although no clinical signs of HSV-incited illness were evident in the G207 administered animals, increased levels of anti-HSV antibodies were reported.

Based on these safety and efficacy reports, G207 was moved into a Phase I clinical trial (Markert *et al.*, 2000a,b). Twenty-one patients with recurrence or progression of malignant glioma after standard therapy were enrolled. All patients had to have undergone prior external beam radiotherapy (minimal dose 5000 cGy) in addition to either craniotomy and surgical debulking (seventeen patients) or biopsy alone (four patients). Ten patients also had been given one or more chemotherapeutics. A Karnofsky performance score of $\geq$70 were required for entry. Primary tumor histologies included fifteen glioblastomas, one gliosarcoma, four anaplastic astrocytomas, and one anaplastic mixed glioma.

This dose-escalation study was intended to determine the maximally tolerated dose (MTD) and any dose-limiting toxicities of G207. Patients were allocated to cohorts by dose level (three patients per cohort); a standard dose escalation scheme using half-log increments was used, with a maximal achievable dose of 3×10^9 pfu established as the ultimate upper limit of G207 to potentially be administered. A waiting period of 10 days between each patient within a cohort and of 28 days between each cohort was included to monitor signs of acute toxicity and/or the development of encephalitis. All patients were given one intratumoral injection into the enhancing portion of the tumor (according to MRI or CT scan), except those treated at the highest dose level. All patients treated with this dose, 3×10^9 pfu, had their tumors stereotactically inoculated in five different enhancing tumor loci as demonstrated by pre-operative imaging studies.

The results demonstrate the safety of G207 administration. An MTD was not established, as even with inoculation of 3×10^9 pfu, there were no definitive dose limiting toxicities, nor any requirement for the use of anti-viral drugs. In the few post-treatment histologic specimens available for review due to subsequent biopsy, tumor resection, or autopsy, there was no evidence of encephalitis (determined by H&E staining) or major inflammatory changes observed. Of the nineteen patients who underwent serologic testing, five were seronegative prior to treatment with G207. Of these five, one patient (treated at the highest dose level) seroconverted after inoculation. Thus, the virus was shown to be extremely well tolerated, even at very high doses.

While efficacy was not the primary endpoint of this study, certain findings support the antiglioma effects of G207 in this trial. Efficacy endpoints of average time to progression by MRI was 116 days and overall median survival of 190 days were only modestly above average in this dose escalation study of patient with recurrent tumors. However, eight of twenty patients revealed reduced enhancement volumes of their tumors at one month after treatment, suggesting that treatment at higher doses and perhaps under a different dosing regimen might improve the response to G207 therapy.

In an effort to gain additional data regarding G207 in the treatment of human glioma, a follow-up Phase Ib study was developed. Objectives of the Phase Ib study include validating the safety and tolerability (MTD) of G207 administration via divided escalating doses given both intratumorally and into functional brain adjacent to the resected tumor. This trial was also designed to evaluate the ability of G207 to replicate within the tumor post-inoculation, define the characteristics of G207 replication in these patients (e.g., virus shedding and reactivation, immunogenicity, impact of cell-mediated immune responses), as well as determine the mean time to disease progression and overall survival. Enrollment in this study has been completed, and data analysis is in progress.

A third study of G207 in patients with malignant brain tumors is scheduled to begin at the University of Alabama, Birmingham in January, 2005. This study will examine the effects of post-inoculation radiation on G207 treatment. The study is based on extensive preclinical data which demonstrates that a single fraction of radiation, when administered approximately 24 hours after G207, increases the replication and spread of viral infection within brain tumors as well as many other neoplasms.

Almost simultaneously with the Phase I trials of G207, HSV1716, derived from the parent wild-type strain HSV-1 17+, also underwent clinical trials to evaluate its toxicity in patients with recurrent malignant glioma (Rampling *et al.*, 2000). This single-mutant replication-selective virus has a genetically engineered deletion of 759 base-pairs within both copies of the $\gamma_1$34.5 gene (Fig. 76.1(e)). HSV1716 was demonstrated to be avirulent in SCID mice, and therefore safe enough to pursue as a possible oncolytic vector therapy candidate in 1994 (Valyi-Nagi *et al.*, 1994). Moreover, efficacy studies in animal models supported utilizing HSV1716 as a possible novel treatment approach to malignant gliomas. Based on preclinical safety and efficacy profiles, HSV1716 was pursued as an anti-glioma agent for humans. Safety and toxicity of HSV1716 administration to patients was first examined in a study that enrolled nine total patients, eight of whom were diagnosed with GBM and one with anaplastic astrocytoma (Rampling *et al.*, 2000). All patients had been treated with prior radiotherapy, and had a KPS of at least 60. In addition, all subjects had also undergone previous surgery and six had received chemotherapy at the time of enrollment. Immunohistochemistry of injected regions when available showed no signs of immunoreactivity for

HSV-1, and the only HSV seronegative patient in the trial did not demonstrate seroconversion. No maximum tolerated dose was established because at the highest dose tested, 1×10^5 pfu, HSV1716 was well tolerated, with no evidence of encephalitis.

A second clinical trial examining HSV1716 suggested that replication occurs in at least some of the high-grade gliomas treated with intratumoral injection (Papanastassiou *et al.*, 2002). Twelve patients with KPS between 60 and 90 were enrolled in this study; eleven GBM patients and one anaplastic astrocytoma patient. Of the GBM patients, there was one newly diagnosed patient; the remaining patients were treated at tumor recurrences. Eleven patients had prior surgery, ten patients had prior radiation therapy, and three had been given some previous chemotherapy. For this trial, all the enrollees were inoculated with 1×10^5 pfu HSV1716 intratumorally, and then underwent surgical resections four to nine days afterwards. Again, no acute toxicity was observed, nor was virus administration responsible for any adverse events. Unlike the first study, both seronegative patients became seropositive by the end of the study. In two patients, HSV1716 was recovered from the resected tumors, but semiquantitative PCR detection methods identified the virus within the tumor tissue of ten patients. The results from the two patients supported the possibility of HSV1716 replication within the tumor. The authors concluded that HSV1716 could feasibly replicate in situ within tumors without toxicity in patients with malignant glioma.

Recently, it was reported that tumor tissue from one of the patients inoculated intratumorally with HSV1716 was cultured in vitro and tested for the presence of that virus (Harland *et al.*, 2002). Though none was found, when the cells were re-infected in vitro, a small fraction was found to not undergo lysis. These cells – which themselves began to proliferate – continued to shed HSV716 at low levels. The authors concluded that this suggested the possibility that, in vivo, a similar persistence of HSV may occur, allowing the virus to continue killing tumor cells over extended periods of time. Further studies of HSV1716's efficacy and safety are ongoing. The virus will be injected into the surrounding brain tissue around the tumors after surgical resection, similar to the Phase Ib trial in progress with G207.

In a recently reported study aimed at examining the safety of HSV1716 inoculation into the brain surrounding the enhancing tumor, twelve patients with newly-diagnosed or recurrent malignant glioma underwent resection followed by inoculation of 10^5 pfu of HSV1716 into the tumor bed. No dose limiting toxicities were observed. Three patients remain alive and clinically stable at 15, 18 and 22 months postsurgery and HSV1716 injection (Harrow *et al.*, 2004).

Oncolytic HSV for Non-CNS malignancies

While the concept of genetically engineered oncolytic HSV-1 was originally developed for the treatment of malignant glioma and other CNS tumors, it was soon explored in preclinical models of other difficult to treat tumors, that developed outside the CNS, including neurofibrosarcomas, melanoma, and non-small cell lung carcinoma, as well as cancer of the breast, liver, pancreas, ovary, head and neck, gallbladder, bladder, prostate, stomach, and colon. Of these, HSV1716 has been studied clinically in patients with melanoma, and NV1020 in patient with colorectal metastases to the liver.

HSV1716: melanoma

Genetically-engineered HSV has been demonstrated to be effective as an oncolytic agent against melanoma in a wide variety of preclinical studies (Miller *et al.*, 2001; Miller and Fraser, 2000; Randazzo *et al.*, 1997; Toda *et al.*, 1999) To explore the possible use of HSV in the treatment of metastatic melanoma (Mackie *et al.*, 2001) performed a pilot study in which five patients with stage 4 melanoma underwent inoculation of HSV1716 into subcutaneous melanoma nodules. Two patients received a single injection of 10^3 pfu of 1716, two received two injections, and one received four injections. Flattening of injected nodules was seen in a single patient, and follow-up biopsies demonstrated tumor necrosis in patient receiving multiple injections; no necrosis was seen in control nodules treated with saline. Immunohistochemical staining was positive for the HSV UL42, a 65 kD DNA-binding protein which is essential for HSV DNA replication and virus growth. Patients tolerated the treatment well, and no change in IgG and IgM titers to HSV were seen. No evidence of response was seen in non-treated lesions, however. Further studies of HSV in melanoma have not yet been reported.

NV1020: colorectal metastases

The third conditionally replication competent HSV-1 to be tested in clinical trials as an oncolytic virus was actually the first such HSV ever to be studied in humans. NV1020 is a purified, mapped form of the virus R7020, which was initially tested in humans as a vaccine against wild-type HSV-1 and 2. R7020 is a genetically engineered virus initially engineered as a candidate for prophylactic immunization against HSV-1 and HSV-2 infection (Meignier,

1991) NV1020 was constructed with a deletion of a 15 kbp region encompasses the UL56 gene and the internal inverted long repeat, extending to the promoter regulatory elements of the ICP 4 gene of the HSV-1 viral genome and includes one copy of the $\gamma_1$34.5 gene. Additionally, a 700 base pair deletion of the native thymidine kinase gene is present that also presents expression of the overlapping transcripts of the UL24 region. Because it was originally designed as a vaccine, a 5.2 kb fragment of the HSV-2 DNA encoding HSV-2 glycoproteins G, D, I and a portion of E is included, as well as a copy of the HSV-1 thymidine kinase gene under the α4 promoter. This maintains sensitivity to antiviral agents such as acyclovir. The virus was extensively tested for genetic stability, with no increase in virulence after nine passages in mouse brain. It has been tested for virulence in mice, guinea pigs, rabbits and the sensitive primate species, *Aotus nancymae* (Meignier, 1991; Meignier *et al.*, 1988, 1990). It has been demonstrated to be safe in *Aotus* at doses up to 10^7 pfu by a variety of routes, including intravenous, oral, subcutaneous, and intramuscular. It retains toxicity when administered intracranially, likely due to the retention of an intact copy of the $\gamma_1$34.5 gene.

Safety has been demonstrated in humans at doses up to 10^8 pfu when administered peripherally in the vaccine trial, and while the virus retained immunogenicity in humans, two doses were required to induce antibody formation. R7020 has been examined as a candidate antitumor agent for a variety of non-CNS tumors in a variety of preclinical models, including hepatoma, colorectal carcinoma, head and neck epithelial squamous cell carcinoma and prostate adenocarcinoma xenografts.

A Phase I study of NV1020 has been conducted in patients with colorectal carcinoma metastatic to the liver. NV1020 was delivered via percutaneous hepatic artery infusion in patients with hepatic metastatic colorectal. Three days after infusion, patients underwent surgery for placement of a hepatic infusion pump; at that time, both tumors and non-malignant liver were biopsied. Patients were observed for 28 days before starting regional chemotherapy. Preliminary results presented at the American Society of Clinical Oncology showed no dose limiting toxicities in the first nine patients, treated at three dose levels (1.3×10^6, 10^7, 1.3×10^7 pfu). Some patients did develop fever, nausea, and headache. Viral DNA but not infectious virus was demonstrated by PCR examination of the hepatic venous outflow. Virus was demonstrated by immunohistochemistry in tumor tissue but not normal liver, and all patients demonstrated a decrease in CEA, a marker for colorectal cancer, over a 28-day period (Fong *et al.*, 2002). A report on the complete trial of all twelve patients treated up to a dose of 1×10^8 pfu is expected shortly. A Phase I//II trial is under way which is designed to examine safety and tolerability of NV1020 in this setting, as well as possible synergies with chemotherapy.

HSV and other cancers

While trials have been opened for patients with other cancers including mesothelioma and head and neck cancers, results remain unpublished at the time of this writing.

Oncolytic HSV: current directions

The potential of HSV used alone as an oncolytic virus is clearly illustrated through these trials (Table 76.1). However, it is likely that recalcitrant tumors such as malignant glioma may require a multi-pronged approach for successful treatment. The proven safety of these engineered viruses permits us to consider the use of HSV-1 mutants that serve not only to kill tumors via infecting, replicating and lyzing them, but also by functioning as vectors to deliver antitumor genes. Preclinical studies using engineered HSV-1 mutants in combination with standard treatment modalities are promising. The enhancement of anti-tumor activity of G207 or other HSV-1 mutants (4009, 7020, 3616) with low-dose and high-dose irradiation has been shown in models of malignant glioma as well as other tumors, including hepatoma, radioresistant squamous cell carcinoma (Advani *et al.*, 1998; Bradley *et al.*, 1999), and cervical cancer (Blank *et al.*, 1999). Combination therapy administering both the $\gamma_1$34.5-deletion mutant R3616 followed by ionizing radiation to mice with human U87 malignant glioma xenografts showed significant growth delay of flank tumors and extended survival of animals with intracranial tumors over the use of either treatment alone (Bradley *et al.*, 1999). These findings have also been demonstrated in intracranial U87 tumors treated with G207 followed by treatment with or without radiation (Markert *et al.*, 2000a,b). In at least some models, this interaction is not simply additive but appears synergistic, possibly due to enhanced viral replication and better dissemination of the virus allowing greater reduction of tumor volume after combination therapy. Increased efficacy has been shown when HSV-1 has been given in conjunction with chemotherapeutic agents, although the purported mechanisms of this increased efficacy vary (Chahlavi *et al.*, 1999).

The demonstrated safety and promise demonstrated by the mutant viruses G207, HSV1716, and NV1020 warrants the further investigation of novel HSV-1 gene therapy vectors. Transgene expression of interleukins or cytokines to enhance immune response against neoplastic cells,

Table 76.1. HSV for the oncolytic viral treatment of tumors

VIRUS	Tumor-selective virus-derivative	Engineered mutation(s) to target tumors	Clinical trials	Tumors targeted
Herpes simplex virus-1	**G207**	1. Deletion of both $\gamma_1$34.5 copies 2. *LacZ* insertion in UL39 (ICP6-inactivation)	Phase I, IB completed Phase I XRT trial approved	Glioma
	HSV1716	1. Deletion of both $g_1$34.5 copies	3 Phase I studies, pilot completed	Glioma, melanoma
	NV1020	1. Deletion, 15 kbp region (UL56 gene to internal ILR) 2. Insertion of HSV-2 gG, gD, gI, partial gE 3. Insertion of HSV-1 tk under alpha-4 4. 700 kbp deletion of UL23-UL24	Phase I completed, Phase I/II underway	Colorectal metastases

anti-angiogenic factors to impede growth of vascular supplies to tumors, or suicide genes to augment the cytotoxic effects of chemotherapeutic agents are all possibilities under study that may demonstrate improved efficacy against human malignancies. Thus, the use of HSV as a portion of a multi-modality treatment regimen involving radiation, chemotherapy and gene therapy, as well possibly oncolytic viral therapy, may hold an important place in the future treatment of patients suffering from these conditions.

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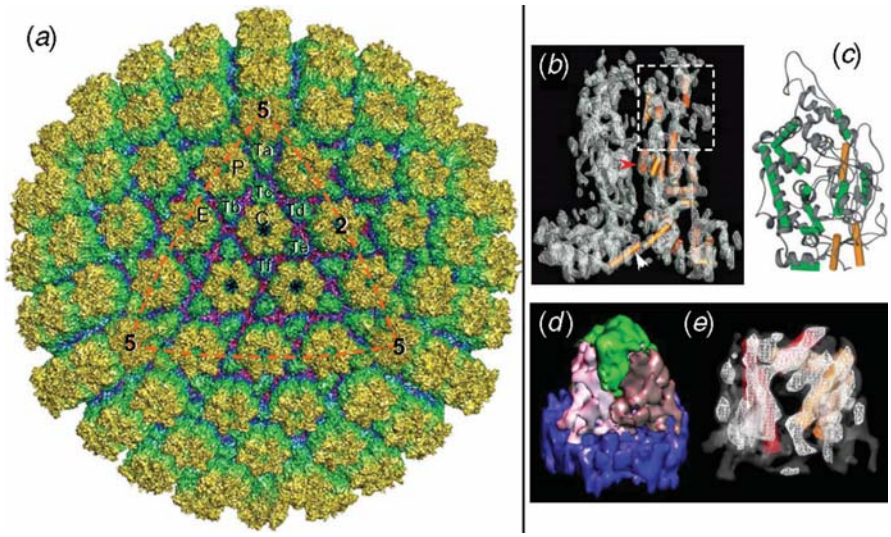


Figure 3.3. Herpesvirus capsid at 8 Å resolution (Zhou *et al.*, 2000) and atomic model of upper domain of the HSV-1 major capsid protein (MCP), VP5 (Bowman *et al.*, 2003). (*a*) Radially color-coded surface representation of the herpes simplex virus type 1 B capsid structure at 8.5 Å. One of the 20 triangular faces is denoted by dashed triangle. The penton and three types of hexons are indicated by '5', P, E and C. Also labeled are the six quasi-equivalent triplexes, Ta, Tb, Tc, Td, Te, Tf. (*b*) Two hexon subunits were shown in wire frame representation with α helices identified in one of the VP5 subunit illustrated by orange cylinders (5 Å in diameter). The red arrowhead points to the 7 helix bundle in the middle domain and the white arrow identifies the long helix in the floor domain that connects adjacent subunits. (*c*) Ribbon representation of the atomic structure of the HSV-1 MCP upper domain determined by X-ray crystallography (Bowman *et al.*, 2003). The helices identified in the hexon VP5 subunit in the 8.5 Å HSV1 capsid map (Zhou *et al.*, 2000) are shown as cylinders: those in green match with helices present in the X-ray structure and those in yellow are absent in the X-ray model, suggesting possible structural differences of MCP packed in the crystal and inside the virion. (*d*) One single triplex is shown as shaded surface representation with individual subunits in different colors: VP19c in green and the two quasi-equivalent VP23 subunits in light and dark grey, all situated on the capsid shell domains of VP5 (blue). (*e*) α-helices identified in the two quasi-equivalent VP23 molecules (in red and yellow cylinders of 5 Å diameter, respectively). Adapted with permissions from publishers.

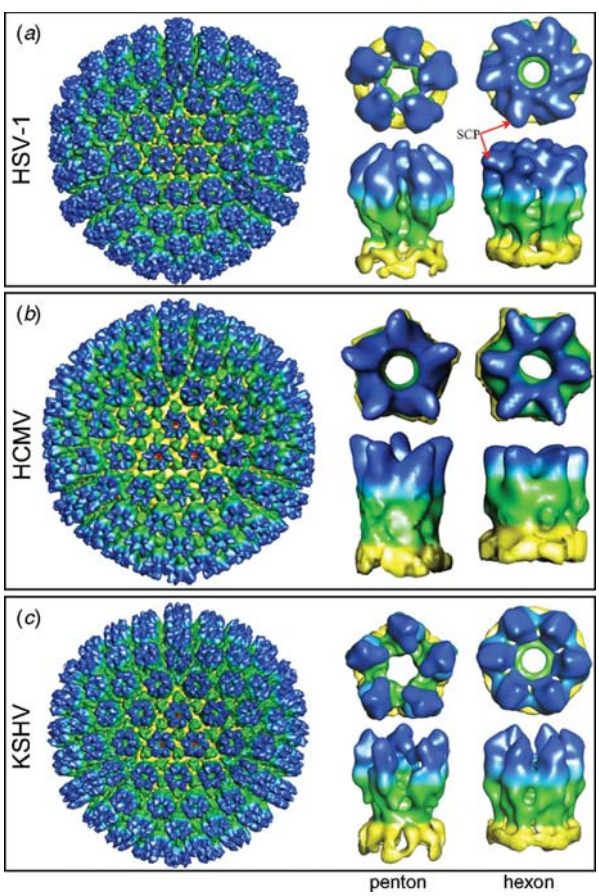


Figure 3.4. Comparison of the three-dimensional structures of alpha, beta and gammaherpesvirus capsids. The capsid maps of HSV-1 (*a*), HCMV (*b*) and KSHV (*c*) are shown as shaded surfaces colored according to particle radius and viewed along an icosahedral three-fold axis. The resolution of the HSV-1 and KSHV capsid maps is 24 Å and that of the HCMV capsid (Butcher *et al.*, 1998) is 35 Å. The right two columns are detailed comparisons of a penton and an E hexon, which were extracted computationally from each map and shown in their top and side views.

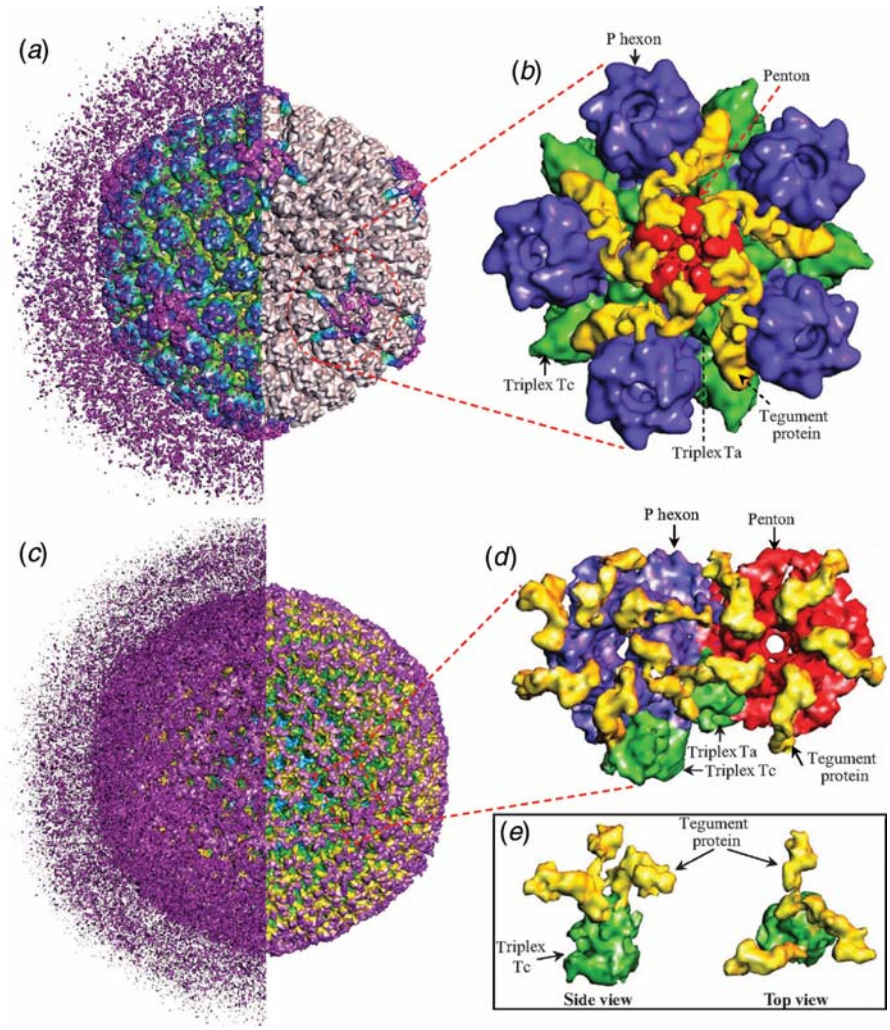


Figure 3.6. Difference of the anchored tegument proteins between HSV-1 ((*a*) and (*b*)) and HCMV ((*c*)–(*e*)). ((*a*) and (*c*)) Radially color-coded shaded surface views of the three-dimensional reconstruction of HSV-1 (*a*) and HCMV (*c*) virions as viewed along an icosahedral three-fold axis. The bulk of the tegument components and the viral envelope are not icosahedrally ordered or polymorphic, thus appearing as disconnected low densities in the icosahedral reconstruction. These disconnected densities were masked out for the right hemisphere to better reveal the icosahedrally ordered tegument proteins, which are shown in blue to purple colors in (*a*) and in purple in (*c*). ((*b*) and (*d*)) Close-up views of the region indicated in (*a*) and (*c*), respectively, showing the molecular interactions of the tegument proteins (yellow) with the penton (red), P hexon (blue) and triplexes (green). In HSV-1, contrary to the extensive tegument association with all hexons, the tegument densities do not interact with any hexon. (*e*) Extracted triplex HCMV Tc with its attached tegument densities. Three tegument densities interact with the upper domain of each triplex (insert Table 3.1).

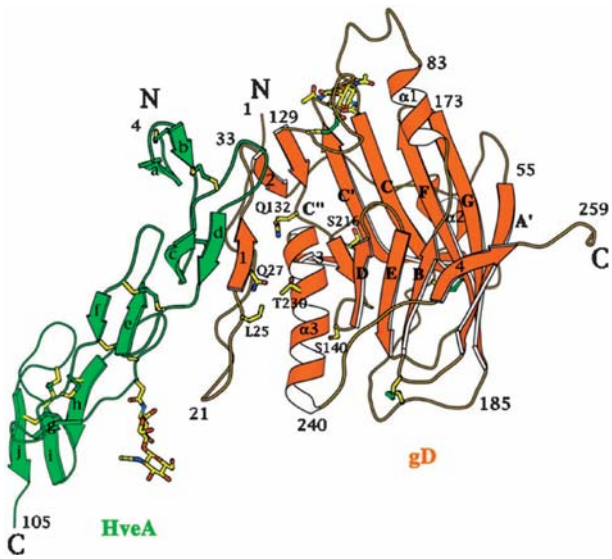


Figure 7.3. Ribbon diagram of the 3D structure of a soluble truncated form of gD (gD285t, colored in orange) bound to HVEM receptor (HveA, colored in green) as determined by X-ray crystallography. The N-terminus (residues 1–37) of gD is devoid of a specific structure when in the unbound state, but folds into a hairpin when bound to HVEM receptor. The β-strand formed by residues 27–29 (indicated with number 1) forms an intermolecular β-sheet with HVEM residues 35–37 (letter d). The core of gD (residues 56–184) has a V-type immunglobulin domain structure, composed of 9 parallel and antiparallel β-strands (letters A to G) that form two opposing β-sheets, and carries an additional α-helix (α1). The residues 185–259 form two α-helices that fold back to the N-ter (α2 and α3), and two β-strands (numbered 3 and 4). The α3 helix supports gD's N-terminal hairpin. An additional β-strand (number 2) is located in the connector sequence (residues 33–55) that precedes the Ig-like core. Reprinted from (Carfi *et al.*, 2001), with permission.

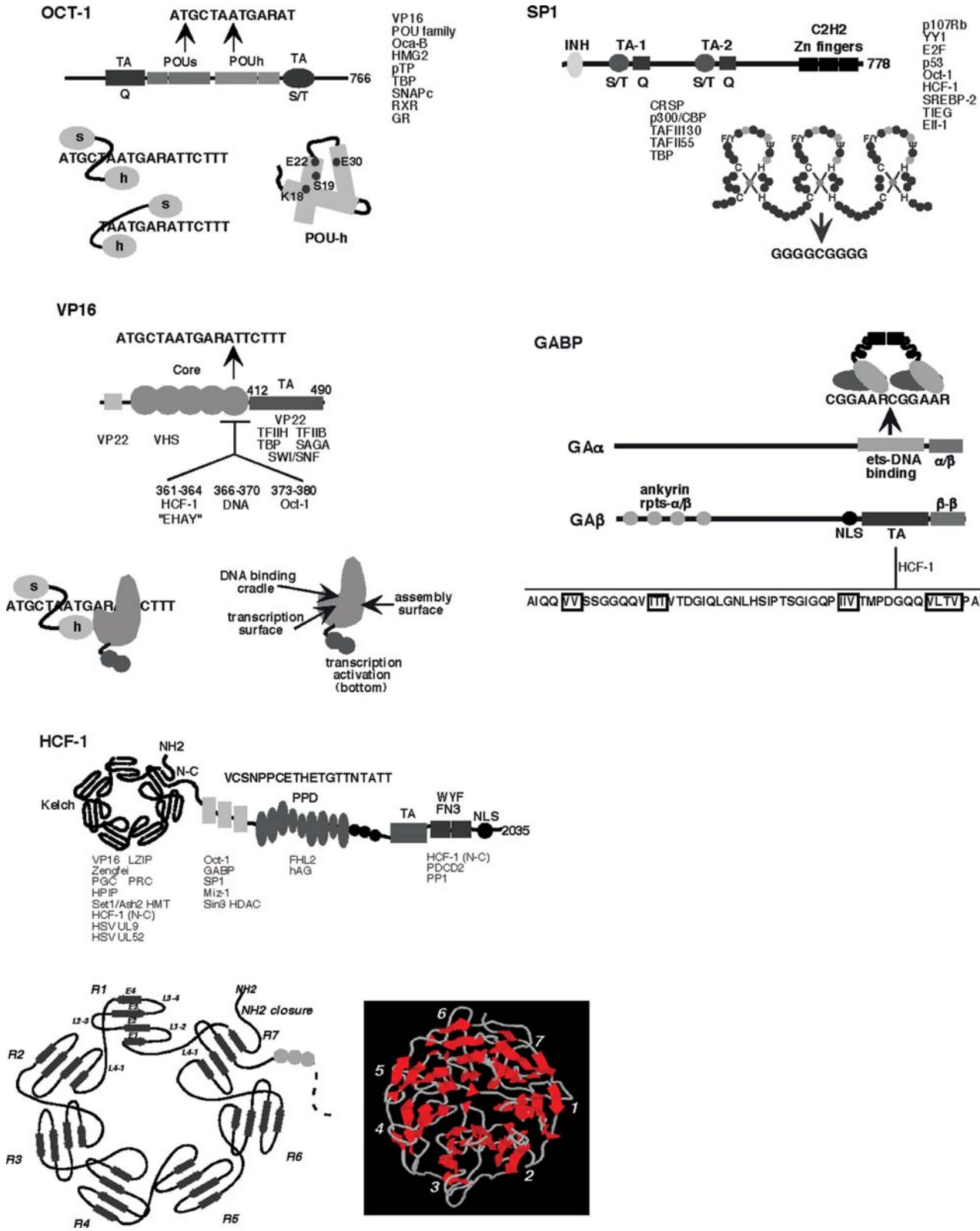


Figure 8.2. The protein components of the HSV IE enhancer complex.
Oct-1: The transactivation (TA-Q and TA-S/T) and POU (POUs and POUh) domains are shown. The POU-specific box recognizes ATGC while the POU-homeobox recognizes TAAT within the enhancer core element (ATGCTAATGARAT). Proteins that bind to the Oct-1 POU domain are listed. The inherent flexibility of the POU domain and the potential orientations of the POUs box in recognition of the core element are depicted. In the schematic representation of the Oct-1 POU-homeobox, the residues which are important for the recognition by VP16 are indicated. (*cont.*)

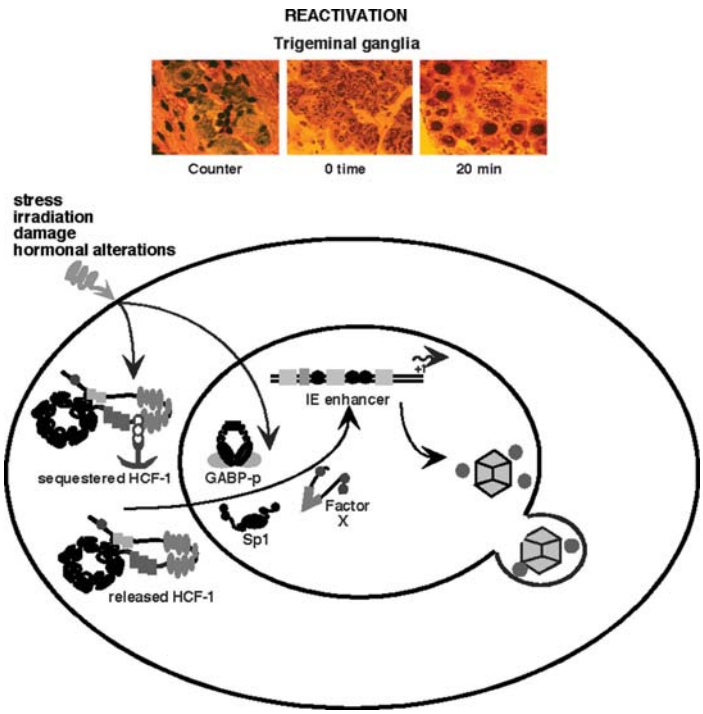


Figure 8.4. Model of the induction of the IE genes during HSV reactivation from latency. (Top) Immunohistochemistry studies of HCF-1 demonstrate that the protein is specifically sequestered in the cytoplasm of sensory neurons (0 time, middle panel) and rapidly transported to the nucleus under experimental conditions that reactivate HSV from latency (explant reactivation stimuli, right panel). (Bottom) Schematic depiction of the activation of IE enhancer components during the initiation of reactivation. Environmental signal(s) result in the release of cytoplasmically sequestered HCF-1 and the activation of DNA binding factors such as GABP, Sp1, or other factors which function in concert with HCF-1 to activate the IE genes and initiate the viral lytic cycle. (see colour plate section)

←

Figure 8.2. (*continued*) *VP16*: The structure and protein interactions of VP16 are represented. The core structure contains the clustered residues that are critical for the assembly of the IE enhancer complex (HCF-1, Oct-1, DNA) while the transactivation domain (TA, aa 412–490) interacts with a number of basal factors and chromatin modifying components. A schematic representation of the VP16 protein structure is shown (left) indicating the various protein interaction surfaces oriented in recognition of the Oct-1 POU-homeobox/ DNA complex.
HCF-1: The amino-terminal kelch, mid-aminoterminal, proteolytic processing (PPD), autocatalytic (Auto), transactivation (TA), WYF-rich, FN3 repeat, and nuclear localization signal (NLS) regions are represented. The proteins that interact with each region are listed below the appropriate domain. The PPD is represented as a series of consensus (large oval) and divergent (small oval) reiterations of the HCF-1 cleavage sequence shown above. (Bottom left) A stylized representation of the HCF-1 kelch domain is shown illustrating the seven predicted blades (antiparallel sheets, E1 through E4; loops, L1–2 through L4–1). For HCF-1, the predicted ring closure utilizes E4 from the animoterminus and E1-2-3 from the carboxyterminus of the domain (NH2 closure). (Bottom right) The derived molecular model of the HCF-1 kelch domain structure is depicted.
Sp1: The inhibitory domain (INH), transactivation domains (TA-1, TA-2), and DNA binding domains (C2H2, Zn fingers) are represented. Proteins or protein complexes that interact with Sp1 are listed. The structure of the C2H2 Zn finger domain is schematically represented: C, cysteine; H, histadine; F/Y, phenylalanine or tyrosine; y, hydrophobic residue. Light circles represent amino acids that are predicted to make DNA contacts.
GABP: The α subunit contains the ets DNA binding domain recognizing the GA box and the heterodimerization domain (α/β). The β subunit contains ankyrin repeats (α/β heterodimerization region), nuclear localization signals (NLS), transactivation domain (TA), and tetramerization sequences ($\beta-\beta$). The sequence of the transactivation domain is shown and the residues that are critical for both transactivation and interaction with HCF-1 are boxed. (see colour plate section)

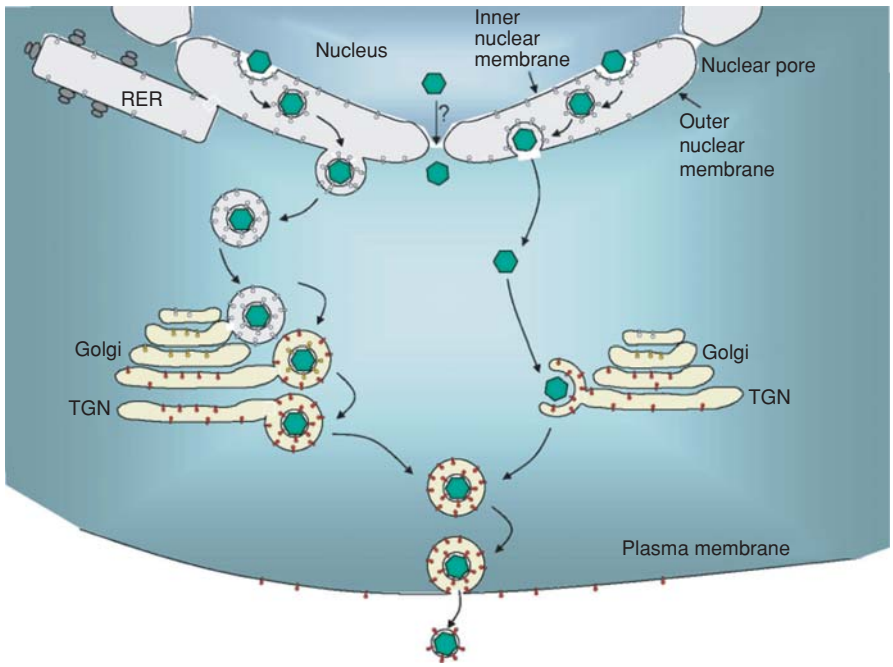


Figure 12.1. Schematic drawing showing the two alternative pathways of alphaherpesvirus egress from infected cells. The single envelopment pathway is depicted to the left, and the double envelopment, or de-envelopment-re-envelopment is depicted to the right of the illustration. The schematic drawing does not shows the gross ultrastructural modifications of the Golgi apparatus and TGN. Perinuclear virions and nuclear membranes are decorated with glycoproteins of different color than virions at the level of the Golgi apparatus and TGN, as well as extracellular virions, to emphasize that the oligosaccharide moieties of the viral glycoproteins are of the immature type in early exocytic compartment, but are of the mature type in the late exocytic compartments and in extracellular virions. The drawing considers also the possibility that nucleocapsids exit the nucleoplasm through modified nuclear pores, without transiting through the perinuclear lumen. (Drawing by courtesy of L. Menotti.)

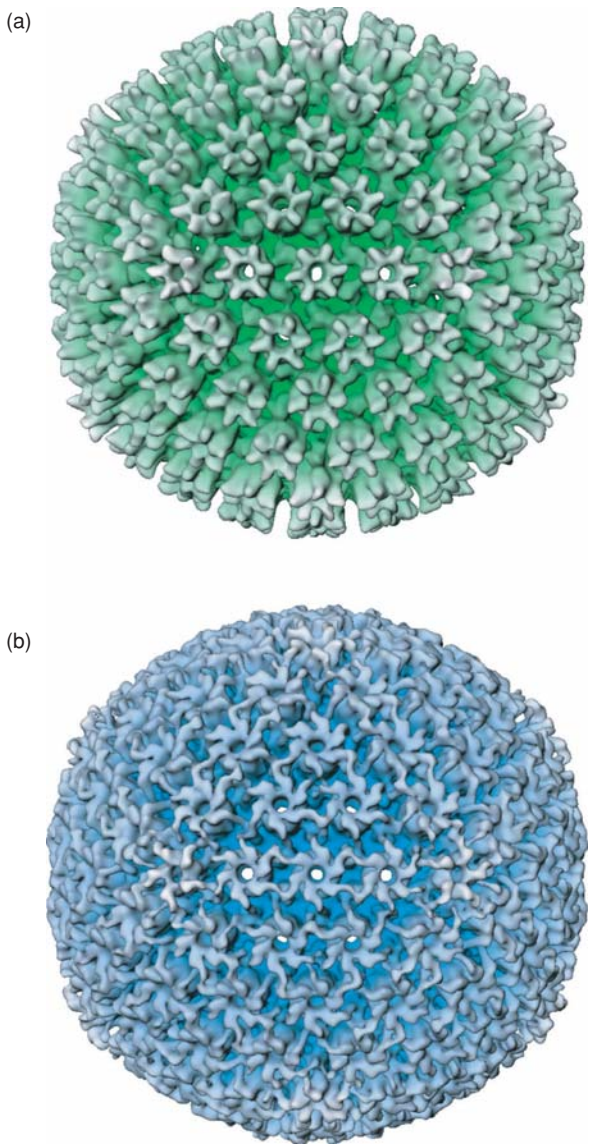


Figure 14.6. Three-dimensional reconstructions of (a) the HCMV B-capsid (adapted from Butcher *et al.*, 1998; with permission from Academic Press), and (b) the HCMV virion (adapted from Zhou *et al.*, 1999; with permission from the American Society for Microbiology). Both structures are viewed along the icosahedral twofold symmetry axes and are radially depth-cued so that darker regions are closer to the centre of the particle and lighter regions are further away.

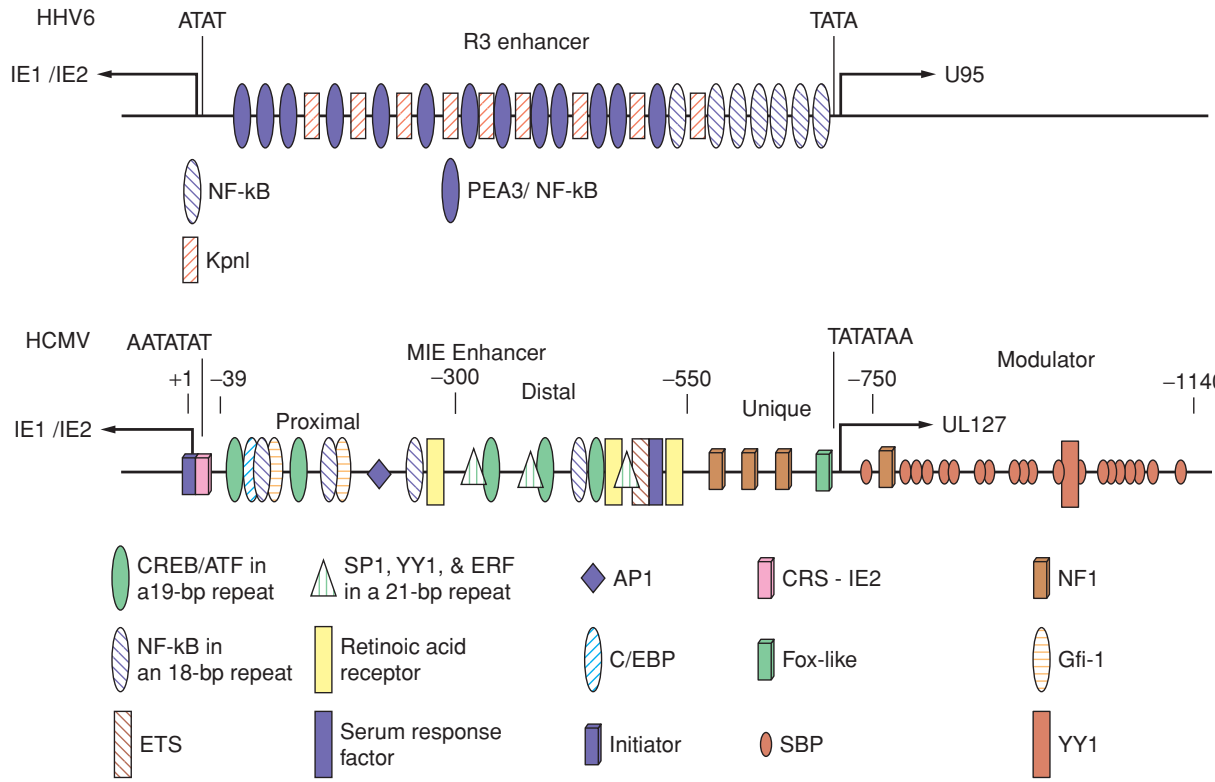


Figure 17.3. Comparison between the HCMV major immediate early (MIE) and the HHV-6 R3 enhancer. Viral genes and promoter/transcription start sites are designated by an arrow. The HCMV also has a unique region and a modulator discussed in the text. The various transcription factor binding sites identified for HCMV and HHV-6 are designated. The AP-2 sites in the R3 enhancer are not identified.

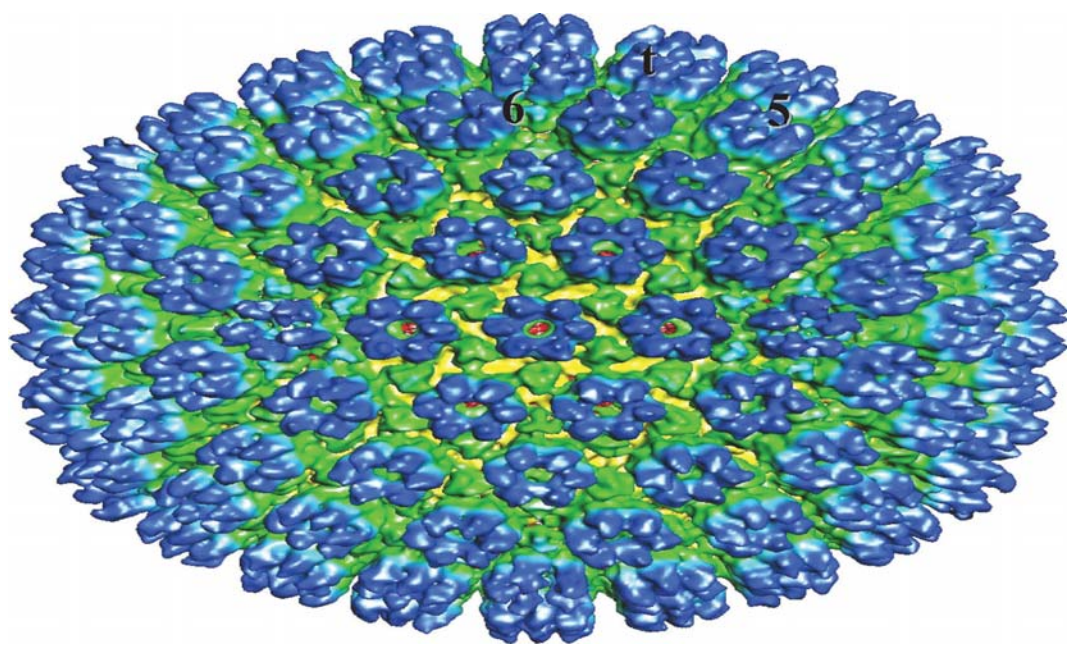


Figure 23.1. 3D structure of the KSHV capsid at 24-Å resolution by electron cryomicroscopy. The capsid is shown as shaded surface color-coded according to particle radius. The three structural component of the capsid are indicated, including 12 pentons ("5"), 150 hexons ("6") and 320 triplexes ("t"). (Wu *et al.*, 2000 with permission).

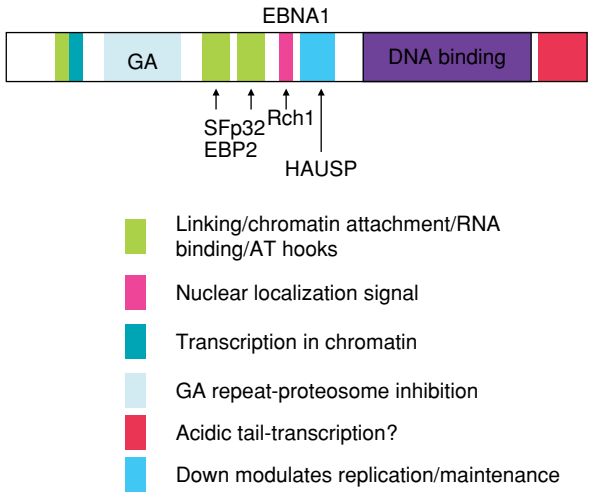


Figure 24.4. Functional protein domains of EBNA1. Amino acid residues are indicated for the boundaries of protein domains for DNA binding, dimerization, chromosome binding sites (CBS), nuclear localization (NLS), or protein–protein interactions (as indicated).

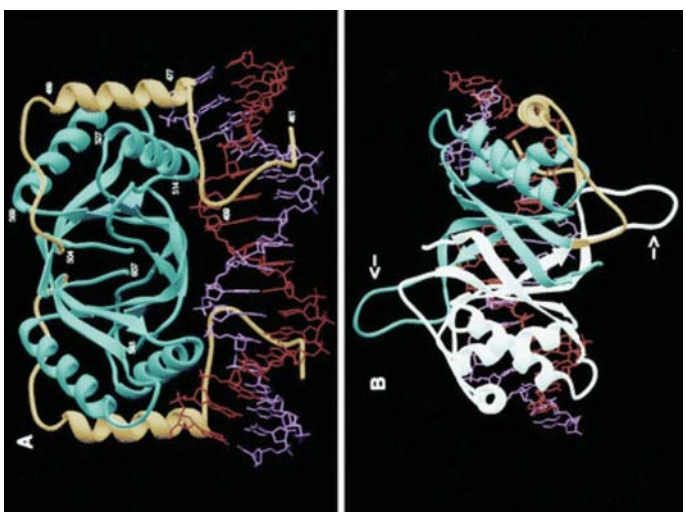


Figure 24.5. X-ray crystal structure of EBNA1 dimer bound to a consensus DNA recognition site (courtesy of Bocharev *et al.*, *Cell* in press). (a) Ribbon diagram showing the core domain (residues 504–607) from each monomer, in blue. Flanking domains are shown in yellow. (b) View down the non-crystallographic axis showing one monomer in white and the other in the same color scheme as used in (a). Proline loops are indicated by arrows.

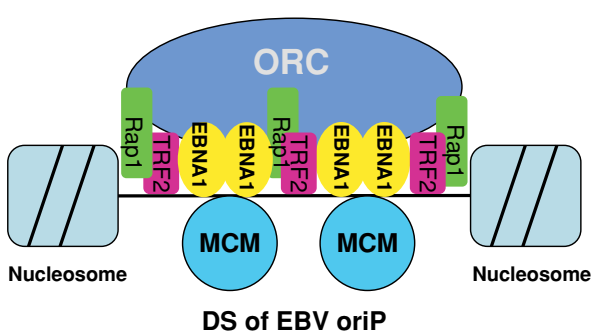


Figure 24.6. Model for *OriP* of EBV. Indicated are protein–protein and protein DNA interactions that are required for the initiation of DNA synthesis at *OriP*.

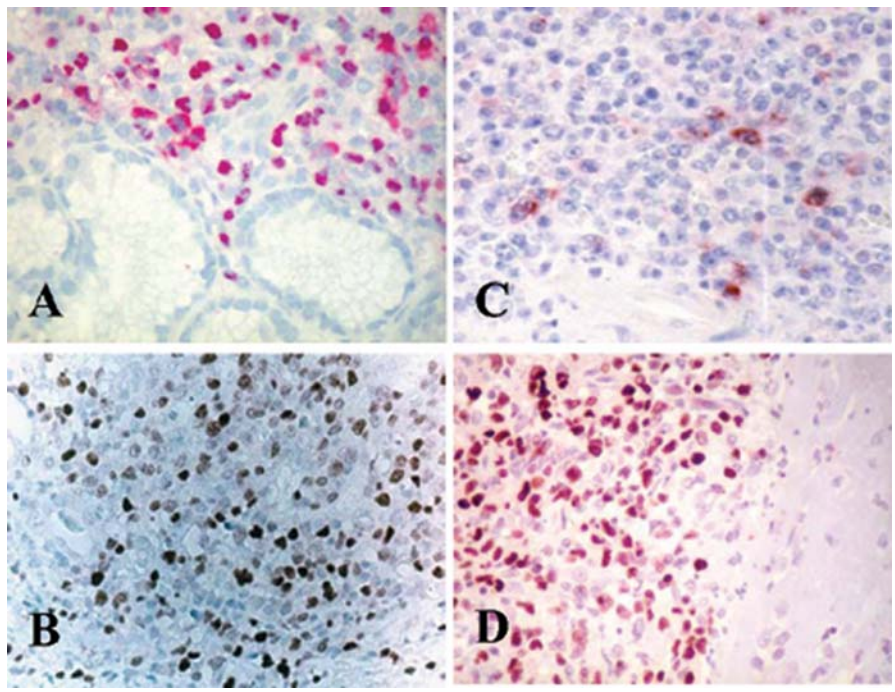
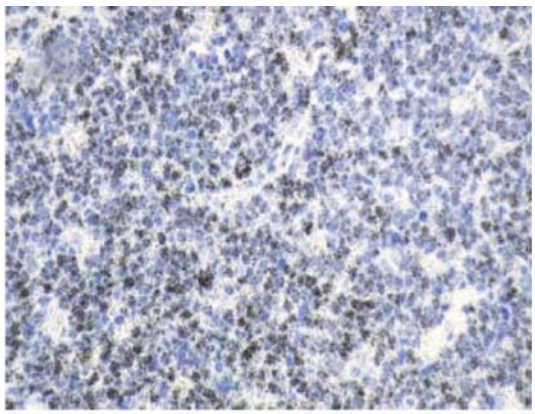


Figure 27.2. Latency III pattern characteristic of the majority of cases of post-transplant lymphoproliferative disease. All known EBV latent genes are expressed in this form of latency: (a) EBERs, (b) EBNA1, (c) LMP1, (d) EBNA2.

(a)



(b)

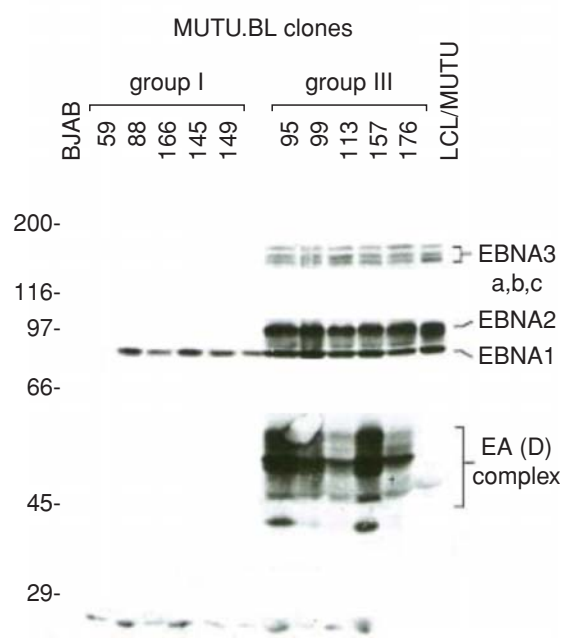


Figure 27.3. Latency pattern characteristic of Burkitt's lymphoma (BL). (a) Expression of EBERs in the tumor cells of BL tissue shown by isotopic *in* situ hybridization and (b) Immunoblotting demonstrating protein expression limited to EBNA1 in so-called 'group I' BL cell lines which recapitulate the in vivo expression profile. 'Group III' BL lines have an expression pattern similar to that of LCLs (Latency III) and represent cell lines that have 'drifted' from a latency I pattern in vitro.

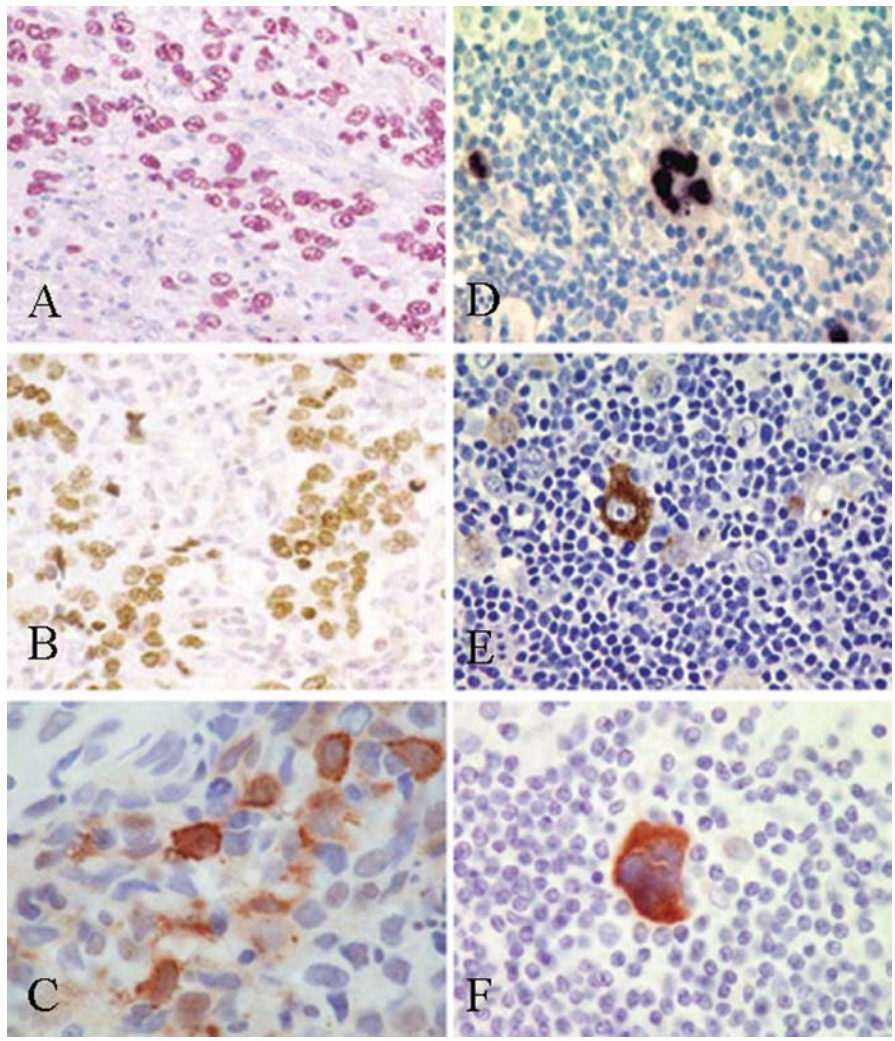


Figure 27.4. EBV latency type II. Left panel shows (a) Expression of EBERs, (b) EBNA1 and (c) LMP1, in the tumor cells of nasopharyngeal carcinoma (NPC). LMP1 expression is not a regular feature of these tumors. LMP2 protein has not yet been reported in NPC tumors, despite the detection of LMP2 RNA. Right panel shows EBV gene expression in the rare tumor cells of (HRS cells) Hodgkin's lymphoma. (d) EBERs, (e) LMP1 and (f) LMP2. EBNA1 protein is also detectable in the majority of cases (not shown). In contrast to NPC, both LMP1 and LMP2 protein are almost always detectable in EBV-infected HRS cells.

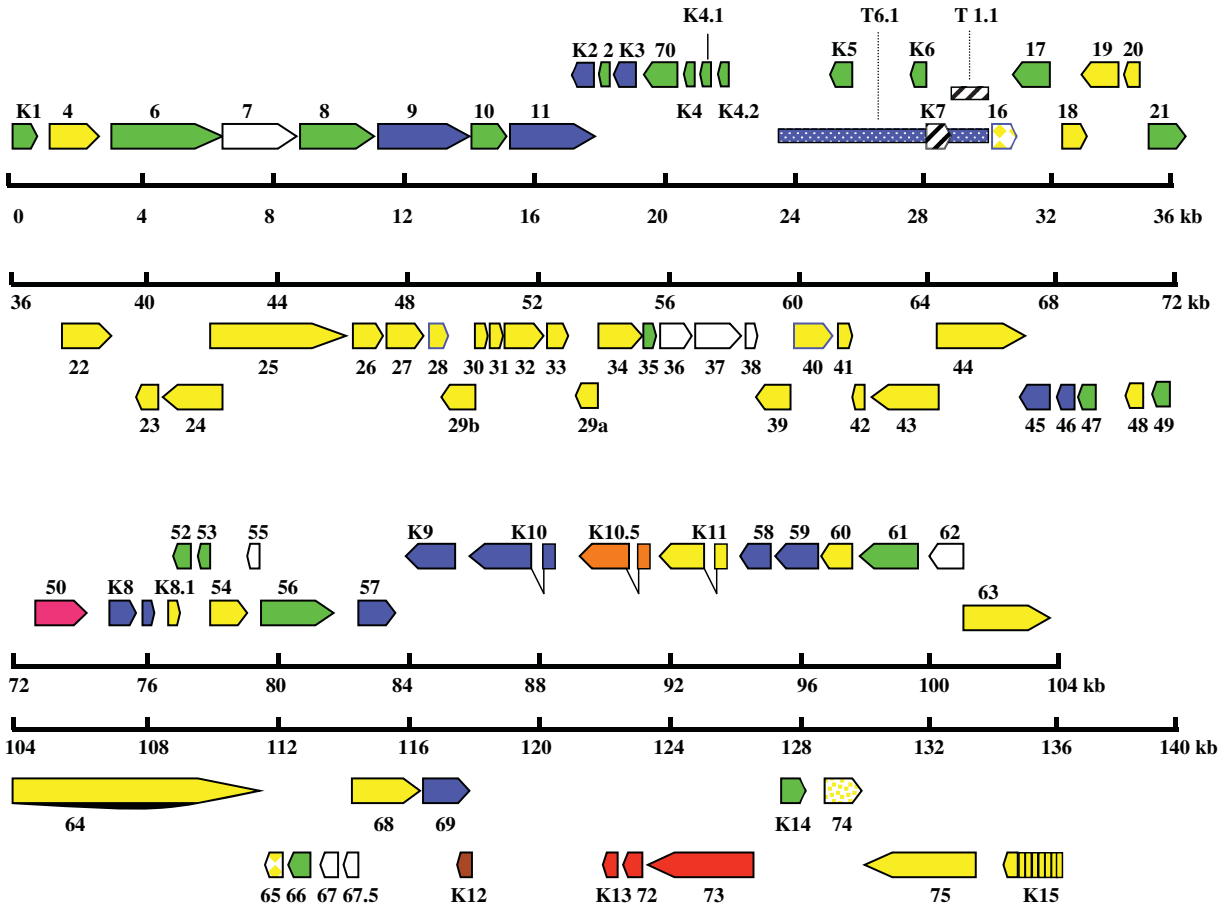


Figure 28.1. KSHV gene expression in PEL cell lines and biopsy samples. This Figure summarizes the expression of individual KSHV genes in PEL cells during latency and following reactivation of the lytic cycle by treatment with TPA, Na-butyrate or heterologous expression of RTA by transfection or transduction. Also included are results from in situ hybridization or immunohistochemistry studies on biopsy samples of KS, MCD or PEL tumors. As discussed in the text, the color-coding is based on a comparison of several reports that studied KSHV genes by Northern blot, real time PCR or DNA array. (See colour plate section)

- Latent gene
- Latent gene in B cells only
- Latent gene in KS spindle cells in vivo; early (in some studies delayed) expression kinetics in PEL cells in vitro
- Immediate–early gene as judged by cycloheximide resistance
- Very rapid onset of gene expression in at least 2 studies
- Early lytic transcript: TPA inducible, unaffected by PFA
- Delayed onset of gene expression
- Late gene expression profile
- Late gene expression profile confirmed by PAA sensitivity
- Discrepant results in different gene array studies

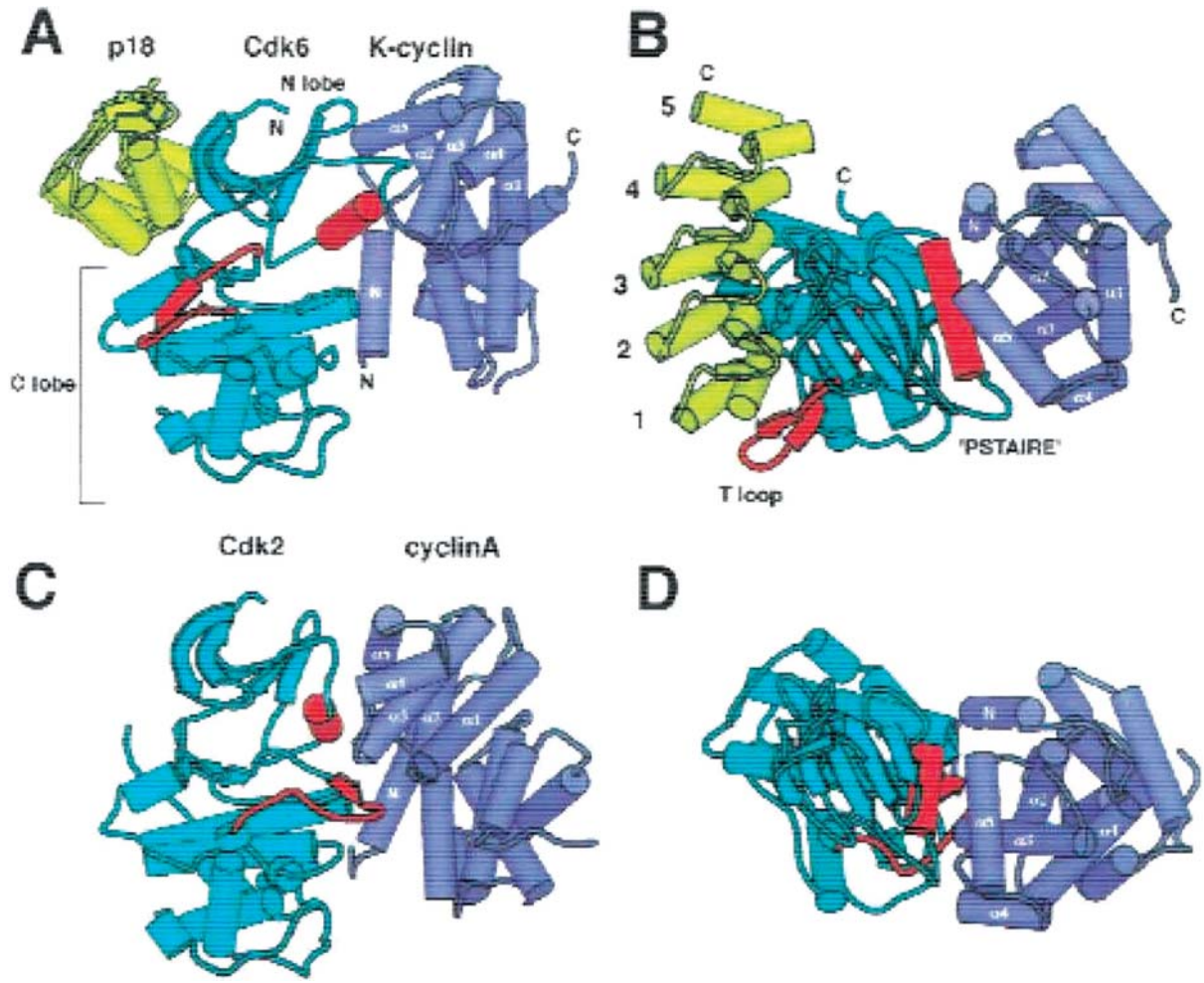


Figure 30.3. The structure of the vCYC (purple), CDK6 (cyan), p18Inkb (yellow) complex from side (a) and top (b) views, compared to cellular cyclin A (purple), CDK2 (cyan) side (c) and top (d) views. Unlike cellular cyclins, the regulatory T-loop of CDK6 is excluded from interaction with vCYC but the PSTAIRE regulatory helix of CDK6 still forms an interface with vCYC, The PSTAIRE helix forms part of the ATP binding domain required for kinase activity while the T loop acts as a negative regulator of kinase activity and must be phosphorylated by cyclin-activating kinases (CAK) in cellular cyclin–CDK complexes. While CAK phosphorylation may enhance vCYC-CDK6 stability, displacement of the T loop by vCYC allows this complex to be active in the absence of CAK activity. The structure of vCYC-CDK6 also reveals loss of the binding pocket used by cyclin-dependent kinase inhibitors (CDKI) of the CIP1/KIP1 family. These and other features support experimental data showing the vCYC-CDK6 not only have a broader target range than cellular D-type cyclins but also escape many normal negative regulatory controls imposed on the cellular cyclin machinery. Reprinted with permission (Jeffrey *et al.*, 2000).

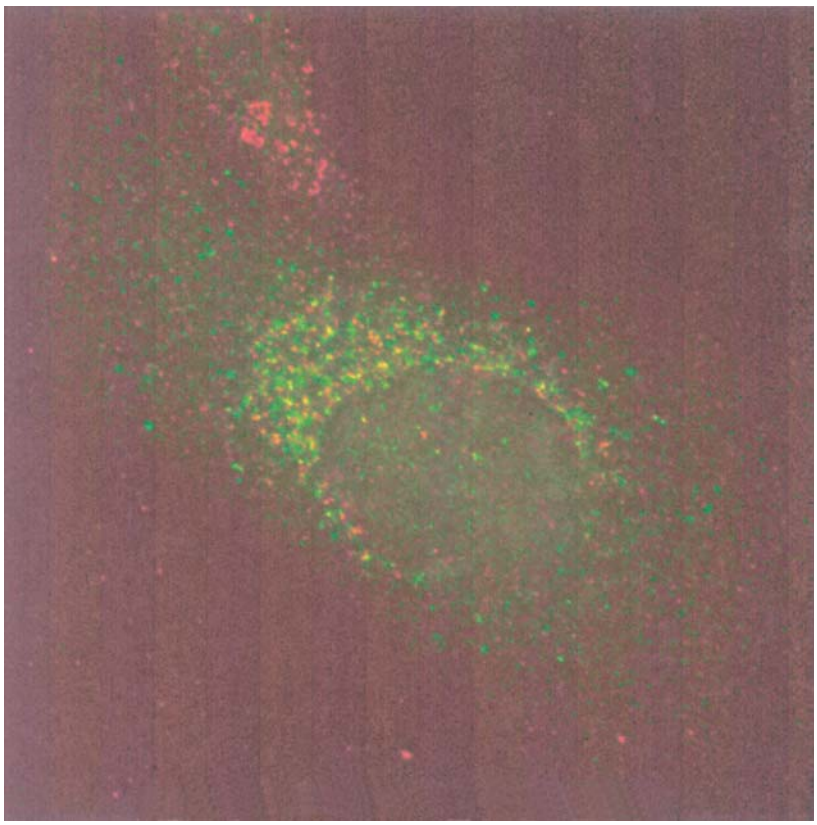


Figure 42.1. Immunofluorescent micrograph of HCMV-infected AEC. Telomerase life-extended human AEC were infected with HCMV. Cells were fixed and stained for the presence of HCMV protein, glycoprotein B (a late product; green) and a cellular marker of the *trans*-Golgi network (TGN46; red).

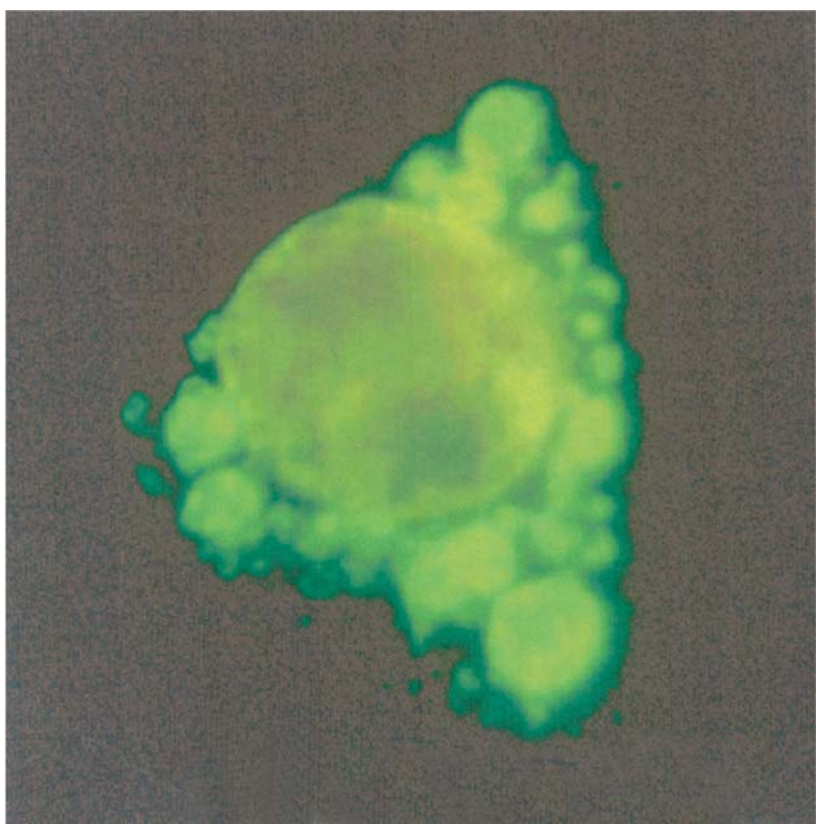


Figure 42.2. Immunofluorescent micrograph of HCMV-infected MDM. MDM were infected with HCMV. Cells were fixed and stained for the presence of HCMV proteins, pp65 (an early product; green) and IE-2 (an immediate-early productl; red).

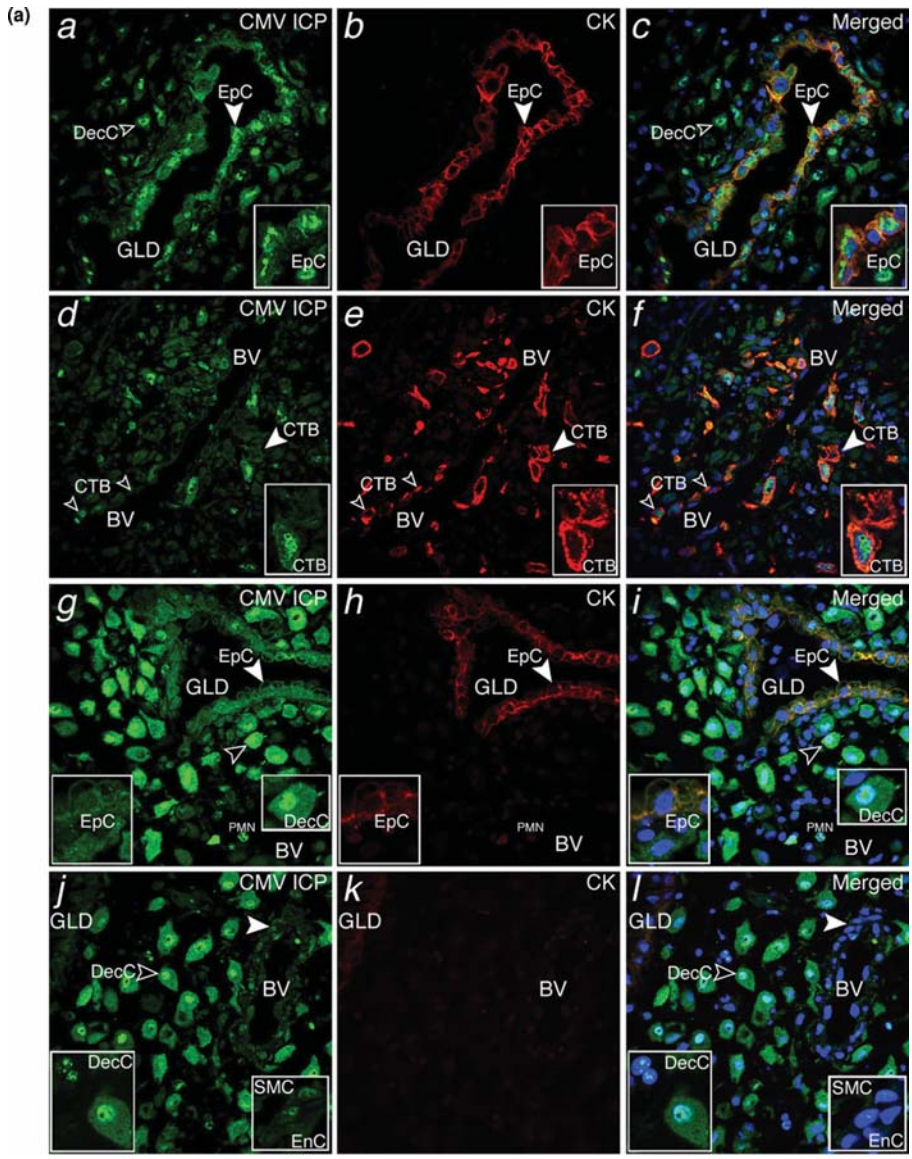


Figure 45.4a. Panel A: CMV replicates in diverse cell types in uterine decidua. CMV infects endometrial glands (GLD), uterine blood vessels (BV), resident decidual cells (DecC) and cytotrophoblasts (CTB) in the decidua. (*a*)–(*c*), Decidual biopsy specimens stained for CMV-infected-cell proteins (ICP, green) and cytokeratin (CK, red), which identified epithelial cells (EpC). (*d*)–(*i*), CMV-infected interstitial and endovascular CTB and DecC. (*j*)–(*l*), Endothelial cells (EnC) and smooth muscle cells (SMC) of uterine blood vessels (BV) are infected. Panel (*b*): Abundant innate immune cells infiltrating the decidua contain CMV proteins. (*a*)–(*c*) CMV gB (green), macrophages (Mφ/DC, CD68, red). (*d*)–(*f*) DC-SIGN+ (green) macrophage/dendritic cells (Mφ/DC) take up CMV gB (red). (*g* (and) *h*) CD56+ (green) natural killer (NK) cells target infection sites. (*i*) DC-SIGN+ cells containing gB. (*j*)–(*l*) Neutrophils (PMN) with phagocytosed proteins from virus-infected cells and endothelial cells (EnC) positive for von Willebrand factor (vWF) in blood vessels (BV). "Merged" indicates colocalized proteins (yellow). Large arrowheads indicate area shown in insets.

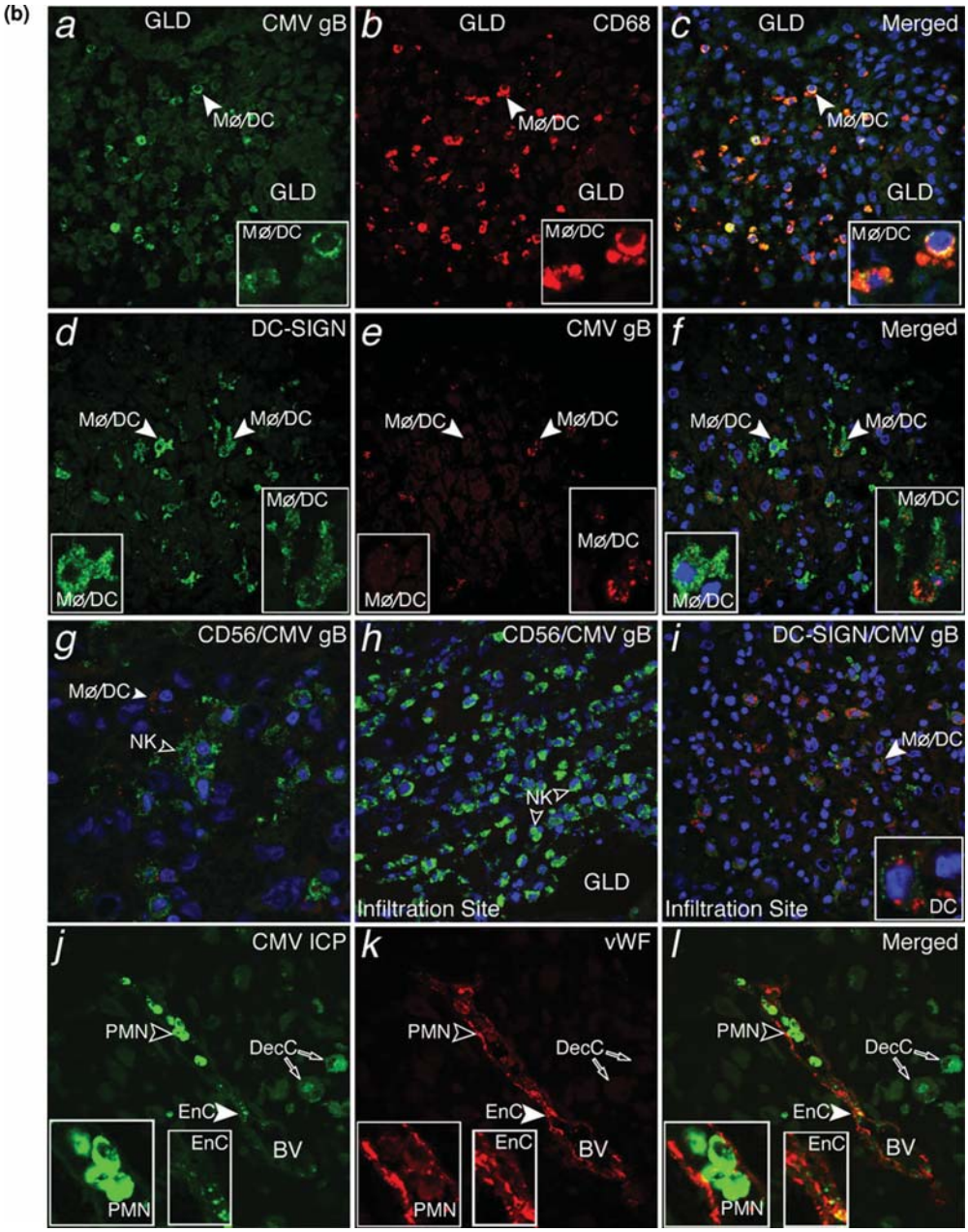


Figure 45.4b. (*cont.*)

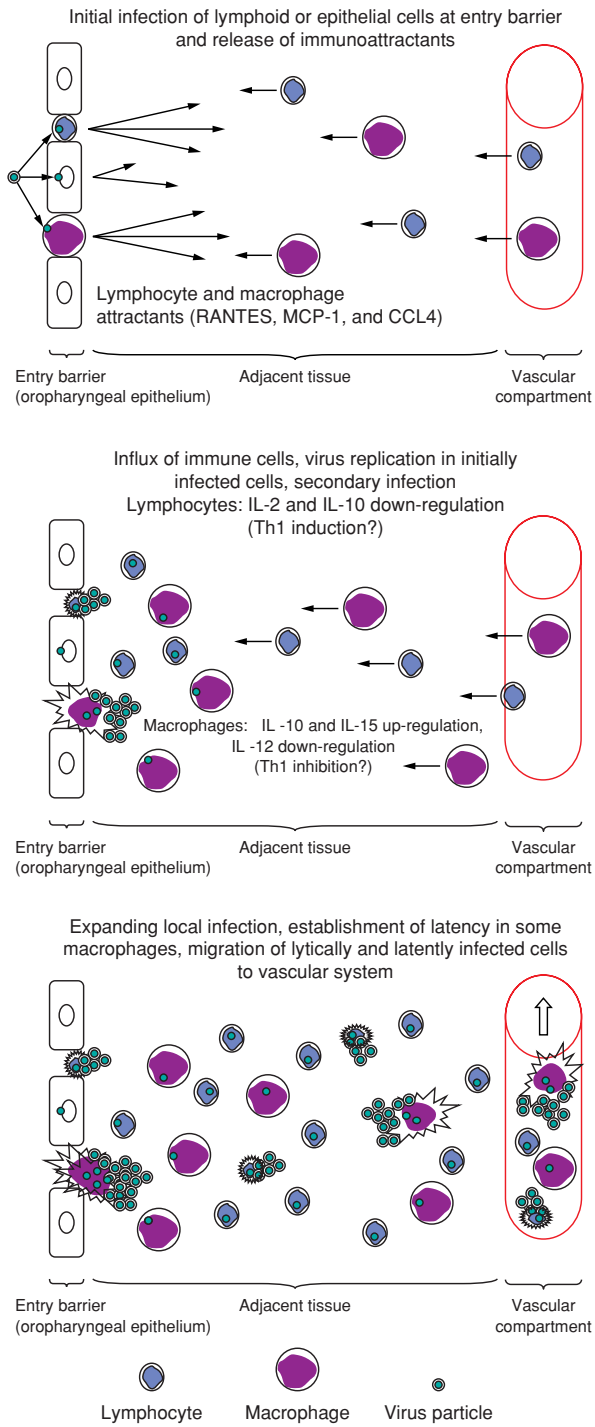


Figure 48.3. Immunobiological events during early primary HHV-6 infection and establishment of latency.

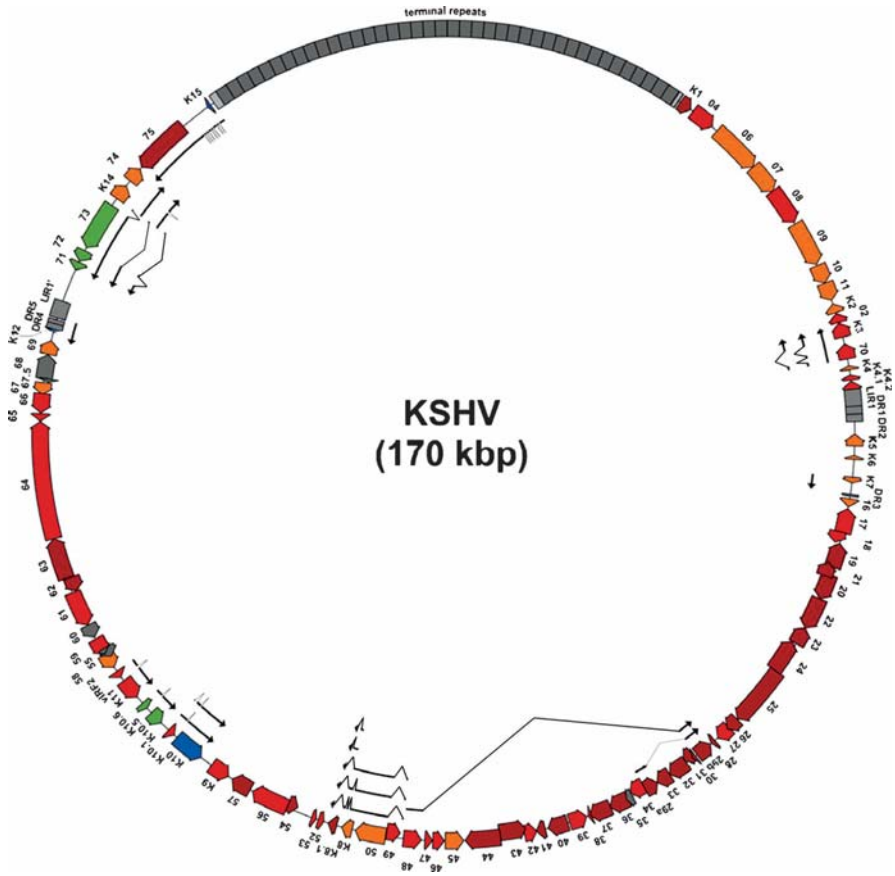


Figure 56.1. Latency genes of KSHV. Transcripts of latent genes are depicted as arrows, superimposed on the physical map of the circular latent viral genome.

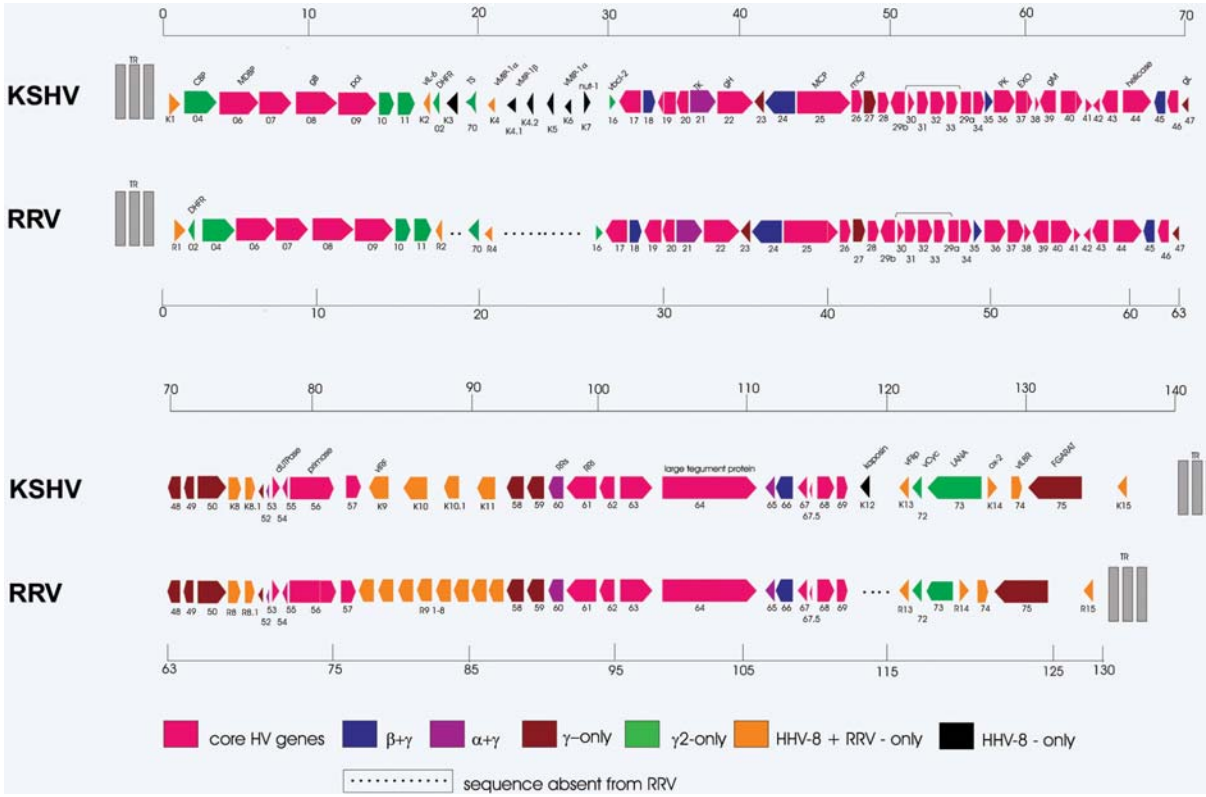


Figure 61.3. Alignment of the KSHV and RRV genomes. The different colors signify ORFs contained in KSHV and RRV 26–95 that are conserved in the indicated herpesvirus subfamilies or subgroups. The square side of the symbol signifies the 5′ end and the pointed side of the symbol signifies the 3′ end of the depicted ORFs. The ORFs are not drawn to scale. (Taken from Alexander *et al.*, 2000, with permission from the *Journal of Virology*.) (see colour plate section)

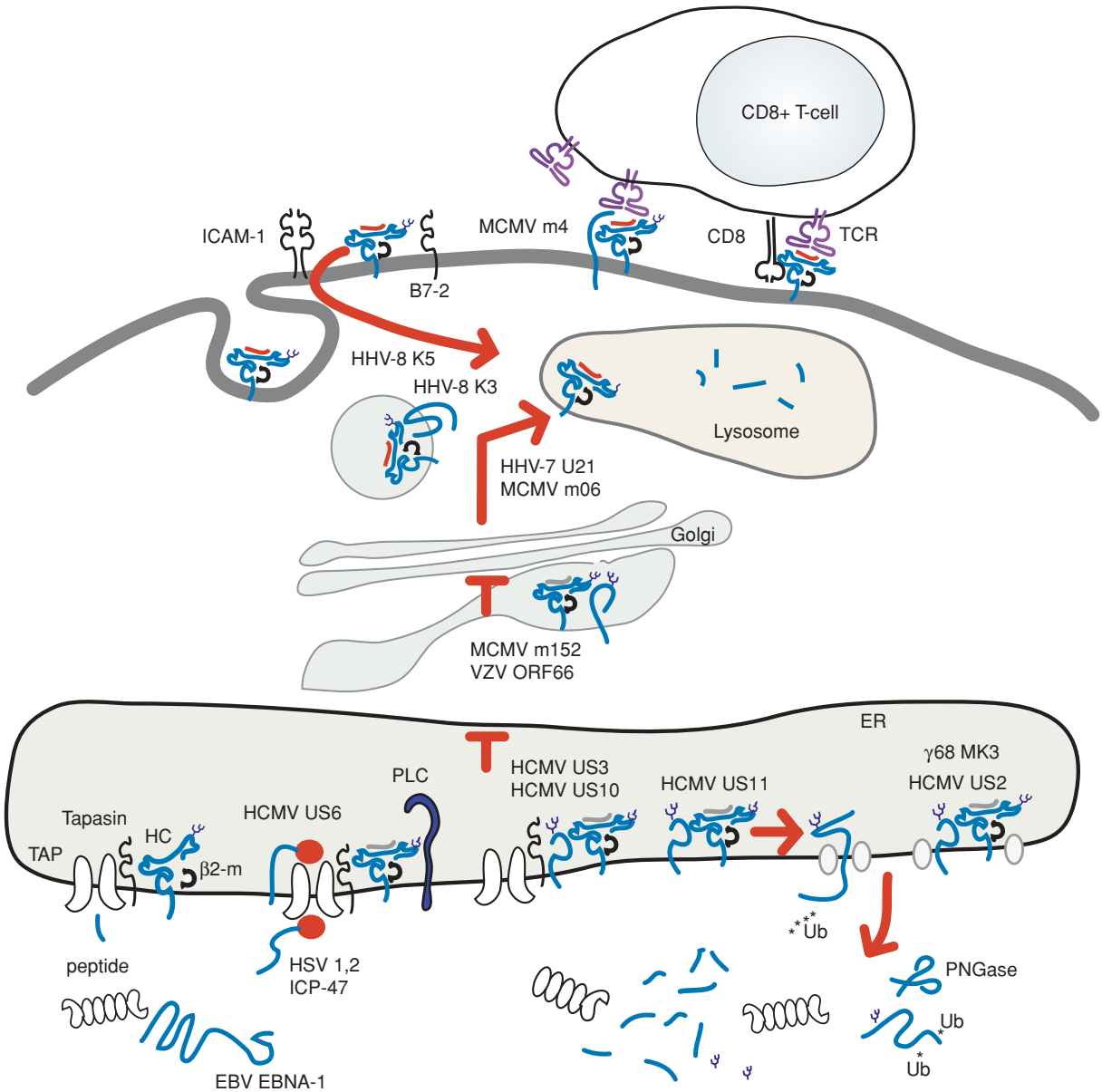


Figure 62.1. Herpesvirus immunoevasins that directly interfere with class I molecule biosynthesis. MHC class I molecules are assembled from free MHC class I HC and β-2 microglobulin within the ER, along with antigenic peptide. Peptides are produced by cytosolic 4m proteasome degradation via its GAr domain. Tapasin and the PLC facilitate loading of peptide cargo onto empty class I molecules. HSV ICP47 and HCMV US6 block TAP peptide transport, while HCMV US3 inhibits tapasin and retains class I complexes in the ER. Following receipt of peptide, the loaded class I molecules travel through the secretory pathway to the cell surface. HCMV US10 delays transport of class I molecules from the ER, while VZV ORF66 and MCMV m152 retain class I in the Golgi complex. HCMV US2, US11 and MHV γ68 MK3 dislocate class I molecules via an unidentified ER membrane pore to the cytosol. The dislocated class I heavy chains are ubiquinated (Ub) and deglycolylated by cellular PNGase prior to proteasomal cleavage. HHV-7 U21, MCMV m6, and HHV-8 K3 redirect class I molecules from the secretory to the endolysosomal pathway for degradation. HHV-8 K5 likewise targets MHC class I, B7-2 and ICAM-1 molecules to the endolysosmal pathway for destruction. MCMV m4 disrupts recognition of cell surface-disposed MHC class I-peptide complexes by the TCR 8of CD8+ T-cells.

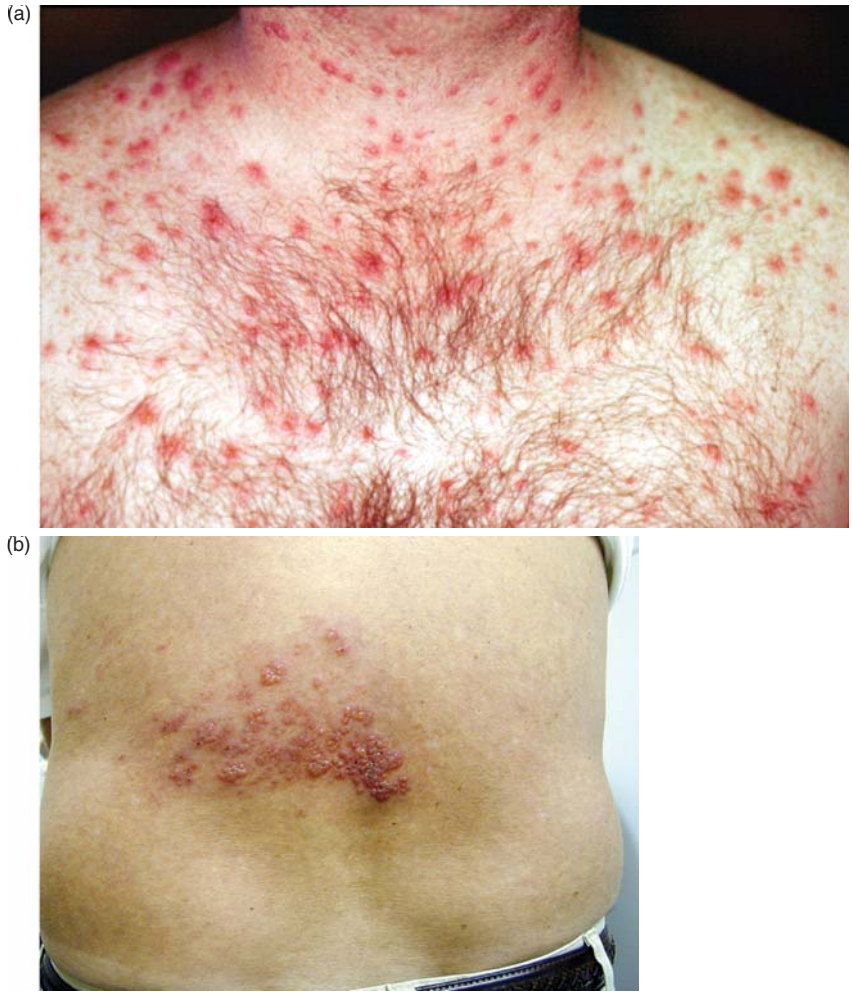


Figure 65.1. Clinical appearance of varicella and herpes zoster. (a) Typical generalized vesicular rash of chickenpox in an adult. (b) Typical dermatomal papulo-vesicular rash of shingles in an adult.

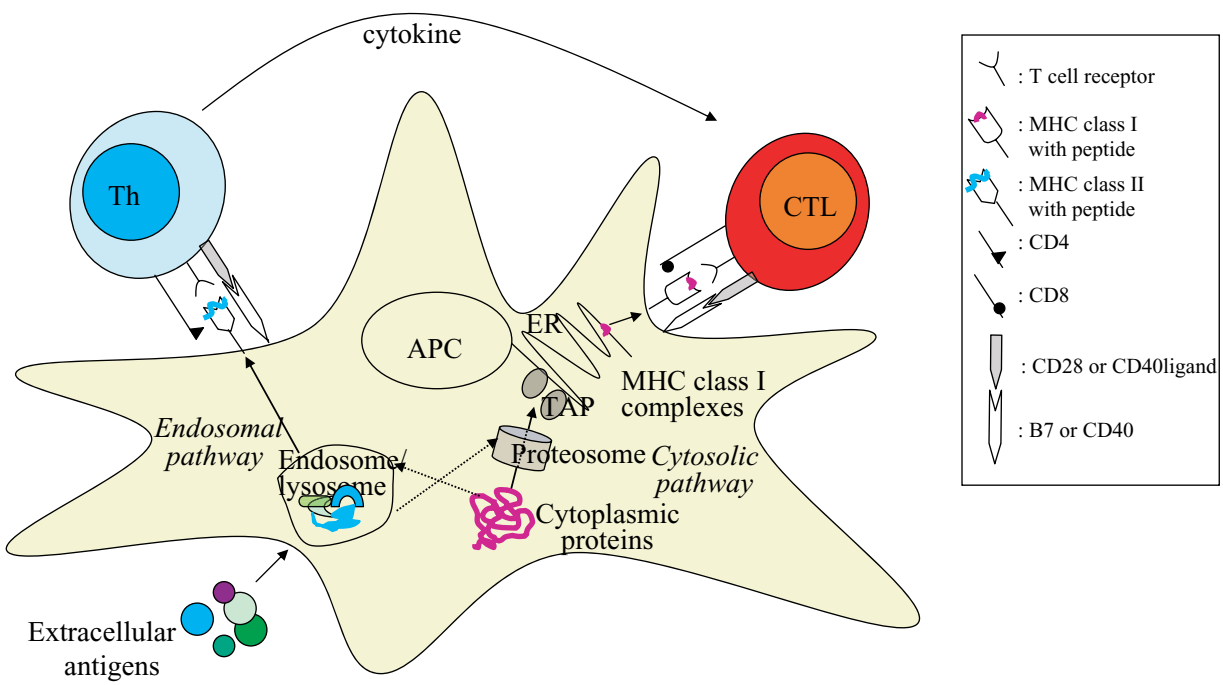


Figure 74.1. Generation of cell-mediated immune response.